REVISED INTERIM WRITTEN DESCRIPTION GUIDELINES TRAINING MATERIALS

Contents

EXHIBIT A

Example 11: Allelic Variants

Specification: The specification discloses a DNA, SEQ ID NO: 1, said to encode a cell surface receptor for adenovirus. The cell surface receptor is designated protein X and its sequence is given as SEQ ID NO:2. The specification states that the invention includes alleles of the DNA that include single nucleotide polymorphisms (SNPs). No allelic sequence information is disclosed, but the specification states that allelic variants of SEQ ID NO: 1 can be obtained, e.g., by hybridizing SEQ ID NO: 1 to a DNA library made from the species of organism that yielded SEQ ID NO: 1.

Claims:

1. An isolated DNA that encodes protein X (SEQ ID NO: 2).

2. An isolated allele of the DNA according to claim 1, which allele encodes protein X (SEQ ID NO: 2).

3. An isolated allele of SEQ ID NO: 1.

Analysis:

Claim 1:

Claim 1 is drawn to the genus of DNAs that encode amino acid sequence SEQ ID NO:2, i.e., all sequences degenerately related by a genetic code table to SEQ ID NO:1. Although only one specie within the genus is disclosed, SEQ ID NO:1, a person of skill in the art could readily envision all the DNAs degenerate to SEQ ID NO:1 by using a genetic code table. One of skill in the art would conclude that applicant was in possession of the

genus based on the specification and the general knowledge in the art concerning a genetic coding table.

Claim 2:

Claim 2 is drawn to a subgenus of allelic DNAs that encode amino acid sequence SEQ ID NO: 2. The specification does not provide any particular definition for the term allele. In this circumstance, the meaning of the term is the ordinary usage in the art. The ordinary meaning of the term allele is one of two or more alternate forms of a gene occupying the same locus in a particular chromosome or linkage structure and differing from other alleles of the locus at one or more mutational sites. See, Rieger et al., *Glossary of Genetics* (1991), p. 16. The alleles in claim 2 are "strictly neutral" because they encode identical proteins, and make no difference to phenotype. See, Rieger et al., p. 17. Although the standard definition refers to genomic sequences and the claims are directed to DNAs, a reasonable interpretation is that the claim is directed to DNAs that include naturally occurring mutational site(s).

The specification discloses only one allele within the scope of the genus: SEQ ID NO:1. The specification proposes to discover other members of the genus by using a hybridization procedure. There is no description of the mutational sites that exist in nature, and there is no description of how the structure of SEQ ID NO: 1 relates to the structure of any strictly neutral alleles. The general knowledge in the art concerning alleles does not provide any indication of how the structure of one allele is representative of unknown alleles. The nature of alleles is that they are variant structures, and in the present state of the art the structure of one does

not provide guidance to the structure of others. The common attributes of the genus are not described. One of skill in the art would conclude that applicant was not in possession of the claimed genus because a description of only one member of this genus is not representative of the variants of the genus and is insufficient to support the claim.

Claim 3:

Claim 3 is drawn to the genus including all DNA alleles of SEQ ID NO: 1. The specification does not provide any particular definition for the term allele. In this circumstance, the meaning of the term is the ordinary usage in the art. The ordinary meaning of the term allele is one of two or more alternate forms of a gene occupying the same locus in a particular chromosome or linkage structure and differing from other alleles of the locus at one or more mutational sites. See, Rieger et al., *Glossary of Genetics* (1991), p. 16. The Rieger reference discloses that there are at least seven different kinds of allele in addition to the "strictly neutral" type discussed above for Claim 2. See, Rieger, pp. 16-17 (amorphs, hypomorphs, hypermorphs, antimorphs, neomorphs, isoalleles, and unstable alleles). The alleles are distinguished by the effect their different structures have on phenotype. According to Rieger, alleles may differ functionally according to their distinct structures. For example, they may differ in the amount of biological activity the protein product may have, may differ in the amount of protein produced, and may even differ in the kind of activity the protein product will have.

The specification discloses only one allele within the scope of the genus: SEQ ID NO:1. The specification proposes to discover other

members of the genus by using a hybridization procedure. There is no description of the mutational sites that exist in nature, and there is no description of how the structure of SEQ ID NO: 1 relates to the structure of different alleles. In addition, according to the standard definition, the genus includes members that would be expected to have widely divergent functional properties. The general knowledge in the art concerning alleles does not provide any indication of how the structure of one allele is representative of other unknown alleles having concordant or discordant functions. The common attributes of the genus are not described and the identifying attributes of individual alleles, other than SEQ ID NO:1, are not described. The nature of alleles is that they are variant structures where the structure and function of one does not provide guidance to the structure and function of others. According to these facts, one of skill in the art would conclude that applicant was not in possession of the claimed genus because a description of only one member of this genus is not representative of the variants of the genus and is insufficient to support the claim.

Conclusions:

Claim 1:

Claim 1 should not be rejected under the written description requirement.

Claim 2:

Claim 2 should be rejected under the written description requirement. An analysis similar to the one set forth above could be used. Since the Office has the burden of presenting evidence to support its position, see

MPEP 2163.04, a reference should be relied on as authority for the Office's interpretation of the claim term "allele."

Claim 3:

Claim 3 should be rejected under the written description requirement. An analysis similar to the one set forth above could be used. Since the Office has the burden of presenting evidence to support its position, see MPEP 2163.04, a reference should be relied on as authority for the Office's interpretation of the claim term "allele."

For the rejections of claims 2 and 3, the Office interpretation of "allele" should be supported by a reference, rather than by taking "notice," because the interpretation is the principle evidence supporting the rejection. See MPEP 2144.03 (For further views on official notice, see *In re Ahlert*, 424 F.2d 1088, 1091 165 USPQ 418, 420 - 421 (CCPA 1970) ("[A]ssertions of technical facts in areas of esoteric technology must always be supported by citation of some reference work" and "allegations concerning specific `knowledge' of the prior art, which might be peculiar to a particular art should also be supported." Furthermore the applicant must be given the opportunity to challenge the correctness of such assertions and allegations. "The facts so noticed serve to `fill the gaps' which might exist in the evidentiary showing" and should not comprise the principle evidence upon which a rejection is based.); see also, *In re Barr*, 444 F.2d 588, 170 USPQ 330 (CCPA 1971) (scientific journal references were not used as a basis for taking judicial notice that controverted phrases were art - recognized because the court was not sure that the meaning of the term at issue was indisputable among reasonable men); *In re Eynde*, 480 F.2d 470, 178 USPQ

Research Article

NGEP, a Prostate-Specific Plasma Membrane Protein that Promotes the Association of LNCaP Cells

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Abstract

Q2 ***NGEP*** **is a prostate-specific gene identified by analysis of expressed sequence tag databases. RNA analysis revealed two spliced forms of NGEP mRNA: a short form encoding a soluble protein (NGEP-S) and a long form encoding a polytopic membrane protein (NGEP-L). Transient expression of myc epitope–tagged NGEP-L showed that it was localized to the plasma membrane. We have now produced a specific antibody to the COOH terminus of NGEP-L and showed that it detects**

Q3 **an ~100-kDa protein in extracts of normal prostate and prostate cancers that contain high levels of NGEP mRNA. The antibody detects a protein that is highly expressed on the apical and the lateral surfaces of normal prostate and prostate cancer cells by immunohistochemistry. The antibody does not detect a protein in the prostate cancer cell line LNCaP, which has very low NGEP mRNA levels. To study NGEP function, two stable LNCaP cell lines were prepared by transfection with NGEP-L and shown to contain similar amounts of NGEP-L protein as human prostate. Confocal immunofluorescence showed that NGEP-L is present on the plasma membrane of the transfected LNCaP cells and is highly concentrated at cell:cell contact regions. Furthermore, as the cell density increased, the cells formed large aggregates. A specific RNA interference that lowered NGEP-L levels prevented formation of cell aggregates. Our results suggest that NGEP-L has a role in promoting cell contact–dependent interactions of LNCaP prostate cancer cells and also that NGEP is a promising immunotherapy target for prostate cancer.** [Cancer Res 2007;67(4):1–8]

Introduction

Adenocarcinoma of prostate is the second leading malignancy in men in United States. It was estimated that in the year 2006 in the United States, 234,460 men would be diagnosed with prostate

FN1 cancer and 28,000 would die of the disease (1).[3] Despite recent advances in diagnosis and treatment, current therapies are unable to completely eliminate the androgen-independent prostate cancer cells that remain after androgen ablation (2–4). To develop improved treatments for prostate cancer, it is important to identify and characterize new molecular targets. Our laboratory has used a computer-based strategy of searching for expressed sequence tags (EST) that are expressed in prostate tissues to identify new genes that are expressed in prostate cancer and not in essential normal tissues (5). One of the genes discovered by this approach is NGEP.

The *NGEP* gene, also now known as TMEM16G, is located on chromosome 2 at 2q37.3. There are two spliced forms of NGEP mRNA. The smaller transcript encodes a 179-amino acid cytoplasmic protein (NGEP-S) and the larger transcript encodes a 933-amino acid polytopic membrane protein (NGEP-L). RNA analysis has shown that NGEP is only detected in prostate samples [normal, benign prostate hyperplasia (BPH), and cancer], indicating it is a differentiation antigen made in normal prostate that continues to be expressed in cancers (1). In an initial attempt to localize the NGEP-L protein, we transfected 293T cells with a myc-tagged NGEP-L cDNA and showed that the protein was localized to the plasma membrane (6). The current study was undertaken to address the question of the location and possible function of NGEP-L in a prostate cancer cell line. Our study shows that in LNCaP cells expressing high levels of NGEP-L, the protein is concentrated at cell:cell contact regions where it seems to promote the association of cells into aggregates and that this aggregation is specifically prevented by RNA interference (RNAi), which dramatically lowers NGEP-L protein levels. This is the first study showing the presence of a prostate-specific protein in the cell:cell contact regions of prostate cancer cells and raises the possibility that NGEP-L may be responsible for cell contact–dependent interactions in the epithelial cells of the prostate.

Materials and Methods

Materials. DMEM and LipofectAMINE were from Invitrogen (Carlsbad, CA). Fetal bovine serum (FBS) was from Hyclone (Logan, UT). Restriction endonucleases and enzymes for molecular biology were obtained from New England Biolabs (Beverly, MA). Fatty acid–free bovine serum albumin (BSA), Triton X-100, octyl glucoside, and protease inhibitors were from Sigma (St. Louis, MO). Tissues were obtained from Cooperative Human Tissue Network (Charlottesville, VA).

Identification of NGEP-L related proteins in the human genome. The NGEP-L related proteins were collected by a BLAST search of a nonredundant human protein database or a BLAT search of a human genome assembly using one NGEP-L protein sequence as a query. Multiple sequence alignment of NGEP-L and related proteins were prepared using T-Coffee (7) and manually adjusted and visualized using CHROMA (8).

Generation of the Rab-Fc-NGEP-L (875–933) fusion protein. The COOH terminus of the NGEP-L (residues 875–933) was expressed in transfected human kidney 293T cells as a fusion protein with a rabbit immunoglobulin (IgG) 1 Fc fragment. The expression vector for the Rab-Fc-NGEP-L (875–933) was produced by subcloning the NGEP-L fragment 875 to 933 in the pSec-Tag Rab-Fc vector using plaque-forming units (Pfu)

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[3] National Center for Health Statistics, Division of Vital Statistics, Centers for Disease Control [Accessed 2006 January]. Available from: http://www.cdc.gov/nchs/nvss.htm.

Polymerase (Stratagene, La Jolla, CA). The PCR products were purified using QIAquick Purification kit (Qiagen, Valencia, CA), digested with *Sac*II and *Xho*I, and cloned in pSec Tag-Rab-Fc digested with *Sac*II and *Xho*I. The plasmids were sequenced in the regions that underwent genetic manipulations. The Rab-Fc-NGEP-L (875–933) plasmid was transfected in 293T cells using LipofectAMINE Plus (Invitrogen) and the manufacturer's protocol. Supernatants from the transfected cells were collected for 5 days beginning 48 h post-transfection and purified on a protein A column (Immunopure Plus protein A, Pierce, Rockford, IL). Protein concentrations were measured by a Coomassie protein assay reagent (Pierce) according to the manufacturer's protocol and checked on SDS-PAGE gels.

Generation of NGEP-L (875–933) 6×-His fusion protein. Expression vectors for the NGEP-L (875–933) 6×-His was constructed by subcloning the NGEP-L (875–933) sequences of human NGEP-L into the pET28a vector (Novagen, Madison, WI) between *Nde*I and *Xho*I sites by overlap extension PCR using *Pfu* polymerase. The vector is designed to introduce a COOH-terminal His_6 tag between the *Xho*I site and the stop codon for affinity purification of expressed proteins.

Escherichia coli strain BL21 (λDE3; Novagen) was used as a host for protein expression. Five hundred milliliters of Luria broth supplemented with 50 μg/mL kanamycin were inoculated with 1 mL of overnight culture grown at 37°C. Cells were grown at 37°C until their absorbance at 600 nm reached ~0.6, and the protein expression was then induced with 0.5 mmol/L isopropyl-1-thio-D-galactopyranoside (Research Products, Mount Prospect, IL). After 4 h, cells were harvested by centrifugation at 5,000 × *g* and 4°C for 10 min. Cells were resuspended in 50 mL of 50 mmol/L Tris-HCl buffer (pH 8.0) containing 50 mmol/L NaCl, 2 mmol/L EDTA, 0.4% (v/v) Triton X-100, 0.4% (w/v) sodium deoxycholate, and 1 mmol/L phenylmethylsulfonyl fluoride (PMSF). After the suspension was sonicated, the inclusion body pellet was obtained by centrifugation at 50,000 × *g* for 15 min at 4°C. The pellet was resuspended in 50 mL of 50 mmol/L Tris-HCl buffer (pH 8.0) containing 50 mmol/L NaCl, 2 mmol/L EDTA, 0.8% (v/v) Triton X-100, and 0.8% (w/v) sodium deoxycholate. After centrifugation at 50,000 × *g* for 15 min at 4°C, the pellet was resuspended in 50 mL of 50 mmol/L Tris-HCl buffer (pH 8.0) containing 50 mmol/L NaCl and 8 mol/L urea. Protein was purified using a Ni-NTA column (Qiagen) according to the manufacturer's instructions. Purity of protein samples determined electrophoretically was >90%. Aliquots of purified proteins were stored at 20°C.

Polyclonal antibody production and purification. One hundred micrograms of Rab-Fc-NGEP-L (875–933) were injected into rabbits with complete Freund's adjuvant for the first immunization and incomplete Freund's adjuvant for subsequent immunizations. Sera were collected after the fourth and sixth immunizations and analyzed by ELISA against the *E. coli*–purified NGEP-L (875–933) 6×-His. An unrelated protein POTE-His, also made in *E. coli*, was used as a control to estimate the nonspecific signal coming from the antisera. Further, to see the cross-reactivity of the antisera, a competition assay was done using Rab-Fc-NGEP-L (875–933). The antibody was further purified by using a Superdex 200 column from which the IgG fraction was collected and concentrated.

Western blotting analysis. Human prostate tissue lysate was prepared by pulverization of the frozen prostate samples in lysis buffer [100 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 2 mmol/L EDTA (pH 8), 0.8% Triton X-100, 0.2% NP40, 1% sodium deoxycholate, 1 mmol/L PMSF, 5 μg/mL aprotinin, 5 μg/mL leupeptin, 1 μg/mL pepstatin]. Tissue and cell debris were removed by centrifugation of the lysate at 14,000 rpm for 10 min. Protein concentration was determined by Coomassie protein assay reagent according to the manufacturer's protocol.

Fifty micrograms of protein extract from different prostate tissues were separated on a 4% to 20% Tris-glycine gel (Invitrogen) and transferred to a 0.2-μm immunoblot polyvinylidene difluoride membrane (Invitrogen) in transfer buffer [25 mmol/L Tris/192 mmol/L glycine/20% (v/v) methanol (pH 8.3)] at 4°C for 2 h at 50 V. Filters were probed with 1:1,000 antiserum or preimmune serum. Primary antibodies were detected with the goat anti-donkey secondary antibody conjugated with horseradish peroxidase (HRP; GE Healthcare Bio-Sciences Corp., Piscataway, NJ) and their respective signals were detected by using an enhanced chemiluminescence detection system (GE Healthcare Bio-Sciences).

Generation of stable cell lines and RNAi. LNCaP cells were grown in RPMI 1640 containing 10% FBS, 1 mmol/L pyruvate, 2 mmol/L glutamine, and 100 μg/mL penicillin and 100 μg/mL streptomycin in a 5% CO_2/95% air humidified incubator at 37°C. For stable cell line generation, the cells at 80% confluency in six-well plates were incubated in serum-free and antibiotic-free RPMI 1640 containing 4 μL LipofectAMINE and 6 μL Plus reagent with 6 μg DNA. Cells were incubated for 4 h at 37°C, after which complete medium was added to bring the final concentration to 10% FBS. Twenty-four hours after the start of the transfection, cells were passaged and split into 10 different plates at different dilutions. At 48 h, the medium was replaced with complete medium containing 750 μg/mL G418 for the selection of the stable clones. Multiple G418 colonies were picked and the clones expressing NGEP-L were selected using Western blot analysis with the polyclonal antibody against NGEP-L.

For NGEP-L RNAi experiments, LNCaP-CL-2 cells were transfected with 200 pmol NGEP-L–specific (D-023184-01) or control small interfering RNAs (siRNA; GL2-Luc; Dharmacon, Chicago, IL) using LipofectAMINE 2000 transfection reagent (Invitrogen) 48 h after plating of the LNCaP-CL-2 cells. Western analysis was done on the cells transfected with the siRNA and the extent of the inhibition of NGEP-L expression was studied. Each transfection was repeated twice in duplicate.

Immunocytochemistry. Cells were plated onto a LabTek chamber slide and grown for 2 days. After 48 h, cells were fixed for 20 min in 4% formaldehyde, treated for 10 min with 0.1% Triton X-100 in PBS, blocked for 30 min with 10% normal goat globulin in PBS, and then incubated at room temperature for 1 h with 1:100 diluted polyclonal antibody for NGEP-L. Subsequently, the cells were incubated at room temperature for 1 h with tetramethyl rhodamine–conjugated secondary antibodies (Invitrogen) at the concentration of 2 μg/mL and then mounted in antifade solution with 4′,6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA). Labeled cells were analyzed by laser confocal microscopy using a ×63 oil immersion objective lens. For labeling of the endoplasmic reticulum (ER) and *cis*-golgi, 1: 100 anti-protein disulfide isomerase (PDI) monoclonal antibodies (mAb; Abcam, Cambridge, MA) and 1 μg/mL anti-human golgin-97, mouse monoclonal CDF4 (Invitrogen) were used, respectively, as the primary antibody. The secondary antibody used for the detection of ER and golgi was Alexa 488–labeled goat anti-mouse antibody (Invitrogen). Q4 Q5

Immunohistochemistry. Paraffin-embedded prostate tissues were cut into 5-micron sections and mounted onto polylysine slides (Histoserv, Germantown, MD). The sections were deparaffinized in xylene followed by graded ethanol hydration into water. The sections were treated with antigen-retrieval solution (DAKO Genpoint, Carpinteria, CA) at 85°C for 20 min. Endogenous peroxidase activity was quenched by incubation with 3% hydrogen peroxidase for 10 min followed by washing in water twice for 5 min. Nonspecific labeling was blocked with 5% BSA in TBS for 20 min. After washing the sections in TBST for 5 min, tissues were incubated with polyclonal antibody against NGEP-L (dilution 1:1,500) in blocking buffer overnight at room temperature. After incubation, the sections were washed twice with TBST for 5 min. Sections were then treated with biotinylated goat anti-rabbit IgG (dilution 1:500; DAKO Genpoint) for 20 min at room temperature, washed once with TBST, and incubated with streptavidin HRP (dilution 1:400) for 30 min. The sections were washed once with TBST for 5 min and the secondary antibody was detected with 3′,3′-diaminobenzidine peroxidase substrate (Sigma). Slides were counterstained with hematoxylin (Histoserv, Gaithersburg, MD), dehydrated in graded ethanol and cleared in xylene, and mounted using Permount (Fisher Scientific, Pittsburgh, PA). Q6

Results

To study the cellular location and function of NGEP-L protein, we generated a specific polyclonal antibody to NGEP-L. Because NGEP-L is a member of the TMEM16 protein family, we used the data of Galindo and Vacquier (9) and two additional members identified in this study to choose a region of NGEP-L that is different from other family members. Our sequence comparison of the TMEM16 family members showed that among the paralogues,

F1 the COOH termini have high sequence diversity (Fig. 1A). Based on this information, we chose residues 875 to 933 to generate NGEP-L antibodies.

Preparation of Rab-Fc-NGEP-L (875–933) protein and generation of the polyclonal antibodies. Rabbits were immunized with a Rab-Fc-NGEP-L (875–933) fusion protein composed of the Fc fragment of rabbit IgG1 and the COOH-terminal amino acids (875–933) of NGEP-L as described in Materials and Methods. Blood was collected from the rabbits after the fourth and sixth immunizations and the antibody titer and specificity were determined by ELISA on plates coated with NGEP-L (785–933) 6×-His protein made in *E. coli* (data not shown). The antibody was purified on a Superdex 200 column to remove albumin and other proteins and the IgG fraction was collected and concentrated.

The minimum amount of recombinant NGEP-L (785–933) that could be detected on a Western blot by the purified polyclonal

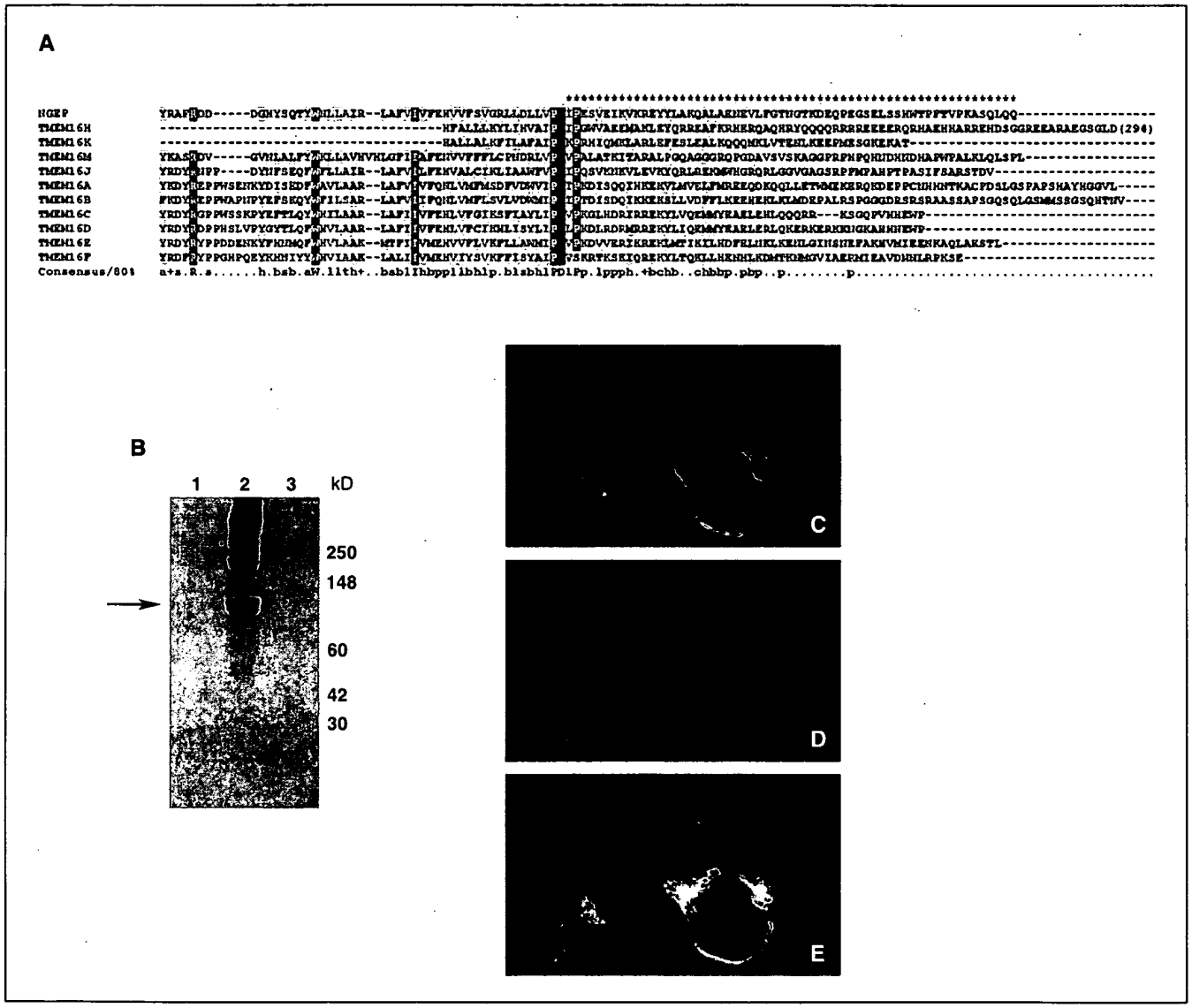

Figure 1. Generation of antibodies against NGEP. *A*, multiple sequence alignment of COOH termini of 11 human NGEP-L related proteins (TMEM16 family). Amino acid residues encoded by exons 22 to 25 of NGEP-L (amino acids 829–933) and its homologous regions in NGEP-L related proteins. TMEM16H and TMEM16K lack the exon equivalent to NGEP-L exon 22. *, peptide sequence used for antibody production. The unaligned residues of TMEM16H are omitted (parenthesis). Markings in the consensus line are as follows: capital letters, amino acid residue identical in 80% or more sequences; *l*, aliphatic (ILV); +, positive (HKR); *t*, tiny (AGS); *a*, aromatic (FHWY); *c*, charged (DEHKR); *s*, small (ACDGNPSTV); *p*, polar (CDEHKNQRST); *b*, big (EFHIKLMQRWY); *h*, hydrophobic (ACFGHILMTVWY). The GenBank gi number and amino acids shown here are as follows: TMEM16A, 40354210:838–960; TMEM16B, 9966845:876–999; TMEM16C, 13899227:887–981; TMEME16D, 30520318:823–920; TMEM16E, 47106048:806–913; TMEM16F, 70778895:808–910; NGEP-L (TMEM16G), 49533623:829–933; TMEM16H, 55741655:851–1232; TMEM16J, 59858803:680–782; TMEM16K, 27545336:332–392; and TMEM16M, 51459226:477–584. *B*, Western blot analysis of NGEP-L–transfected cell extracts with an NGEP-L antibody. The expected 100-kDa protein is detected in extracts transfected with pcDNA3.1-NGEP-L myc (*lane 2*). A cell extract from vector only–transfected cells or protein POTE21-transfected cell produced no signal (*lanes 1* and *3*, respectively). Forty micrograms of protein lysate were loaded onto a 4% to 20% gel and Western blot analysis was done using NGEP polyclonal antibody at a dilution of 1:1,000. HRP-labeled donkey anti-rabbit secondary antibody at a dilution of 1:2,000 was used for detection. *C* to *E*, 293T cells were transiently transfected with cDNA expressing EGFP-NGEP. After 24 h, the cells were stained with anti-NGEP antibodies. The fluorescence of EGFP-NGEP in the 293T cells expressing the fusion protein (*C*; *green*) completely colocalized with the anti-NGEP (*D*; *red*) as shown in (*E*; *yellow*). Nuclei were stained with DAPI (*blue*).

antibody was determined using different dilutions of polyclonal antibody and different concentrations of NGEP-L (785–933) 6×-His. Using Western blot analysis, the NGEP-L antisera at a dilution of 1:1,000 could detect 1 ng of recombinant NGEP-L (785–933; data not shown). To determine if the polyclonal NGEP-L antibody could detect NGEP-L protein in mammalian cells, 29f3T cells that do not express NGEP-L were transfected with a pcDNA3.1/NGEP-L-myc plasmid. Total lysates were subjected to SDS-PAGE. As shown in Fig. 1*B*, the polyclonal antibody specifically detected a strong band of ~100 kDa in 293T cells transfected with pcDNA3.1 NGEP-L-myc. It also detected higher molecular bands very likely representing aggregated protein (Fig. 1*B*, *lane 2*). (Aggregation of polytopic proteins is often observed when these proteins are produced at high levels by transfection.) No signal was detected in the untransfected cells (Fig. 1*B*, *lane 1*) or in 293T cells expressing a cDNA encoding a control protein POTE21 (Fig. 1*B*, *lane 3*; ref. 10). These data establish that the polyclonal antibody against NGEP is specific and can detect NGEP-L expressed in mammalian cells.

We showed previously that NGEP-L tagged with myc localizes in the plasma membrane of transfected 293T cells (6). To determine if the polyclonal antibody detects the same protein, we chose to immunologically colocalize transfected NGEP-L by transfecting 293T cells with enhanced green fluorescent protein (EGFP)-NGEP-L and determining if the EGFP signal (green) colocalizes with one produced by an antibody to NGEP-L detected with a tetramethyl rhodamine goat anti-rabbit antibody (red). The images in Fig. 1*C–E* show that the two signals overlap, indicating the NGEP-L antisera detects NGEP-L in transfected cells (Fig. 1*E*). No signal was obtained from the untransfected 293T cells or when prebleed sera was used for the immunofluorescence on NGEP-L–transfected cells (data not shown), showing the antibody is specific to NGEP-L and can detect NGEP-L in formalin fixed cells.

Expression of NGEP in prostate tissue. Because RNA studies have shown that *NGEP-L* is a prostate-specific gene (6), we used the antisera to test for the presence of NGEP-L in protein extracts of normal prostate and prostate cancer and a few normal tissues. Fifty micrograms each of tissue lysates were resolved on a 10% SDS gel, blotted, and then reacted with the polyclonal NGEP-L antibody. As shown in Fig. 2*A*, a band of ~100 kDa was detected in protein extracts of 293T cells transfected with pcDNA3.1 NGEP-L-myc (Fig. 2*A*, *lane 2*) as well as bands in the high molecular weight regions due to aggregated protein. A specific band at the expected M_r of ~100 kDa was detected in tissue extracts from normal prostate (Fig. 2*A*, *lane 3*), *BPH* (Fig. 2*A*, *lanes 4* and *5*), and prostate cancers (Fig. 2*A*, *lanes 6* and *7*). The expression of NGEP-L in one of the BPH samples was low (Fig. 2*A*, *lane 4*). No signal was detected in liver and brain tissue lysates. To evaluate the quality of the lysates, we did a Western blot with a β-actin antibody as shown in Fig. 2*B*; a similar amount of actin was detected in all the tissue lysates. We conclude the antisera can specifically detect NGEP-L in prostate tissue lysates. F2

Generation of NGEP-L–expressing prostate cell line. Previously, we found that NGEP mRNA is present at extremely low levels in LNCaP cells using reverse transcription-PCR. This level is much less than the levels present in tissue and tumor samples from patients, suggesting NGEP-L expression was lost when the LNCaP cell line developed. As expected, attempts to detect NGEP-L protein in LNCaP cells by Western blot were unsuccessful (data not shown).

To study the location and possible function of NGEP-L in prostate cancer cells, we established two stable LNCaP cell lines expressing NGEP-L by transfecting NGEP-L into these cells using a

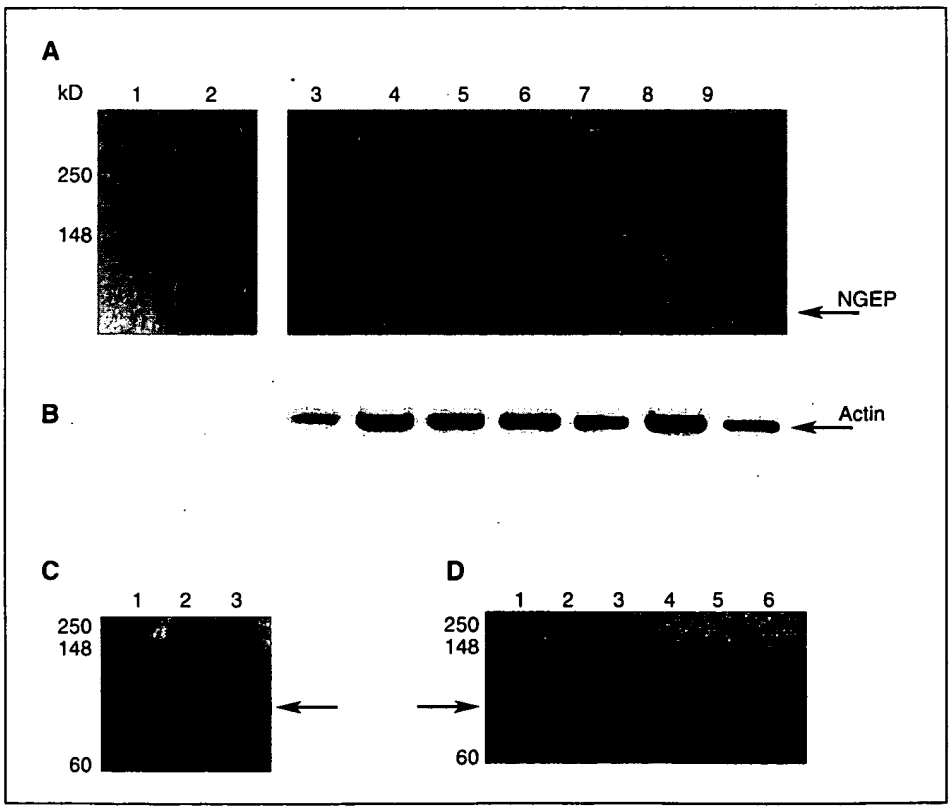

Figure 2. Western analysis of NGEP-L in prostate tissue and prostate cancer cell lines. *A*, Western blot analysis of NGEP-L expression: 50 μg of lysate from 293T nontransfected 293T cells (*lane 1*), 30 μg of cell lysate from 293T transfected with cDNA encoding NGEP-L (*lane 2*), and 50 μg of tissue lysate from normal prostate (*lane 3*), BPH (*lanes 4* and *5*), prostate cancer (*lanes 6* and *7*), brain (*lane 8*), and liver (*lane 9*) were analyzed by Western blotting using rabbit anti–NGEP-L antibody (1:1,000). *B*, the blot was probed with anti–β-actin (1:250; Abcam) as a loading control. *C*, Western analysis of NGEP-L expression in the stable cell lines. The samples (40 μg) loaded are the following: *1*, LNCaP-V; *2*, LNCaP-CL-1; and *3*, LNCaP-CL-2. A protein band ~100 kDa was observed for NGEP-L–transfected cells. *D*, subcellular fractionation was done using differential centrifugation in LNCaP-CL-2 and LNCaP-V. The samples (40 μg cells extracts) loaded are the following: *1*, LNCaP-CL-2 cytosol fraction; *2*, LNCaP-CL-2 nuclear fraction; *3*, LNCaP-CL-2 membrane fraction; *4*, LNCaP-V cytosol fraction; *5*, LNCaP-V nuclear fraction; and *6*, LNCaP-V membrane fraction. NGEP-L is present in the crude membrane and nuclear fractions but not in the cytosolic fraction.

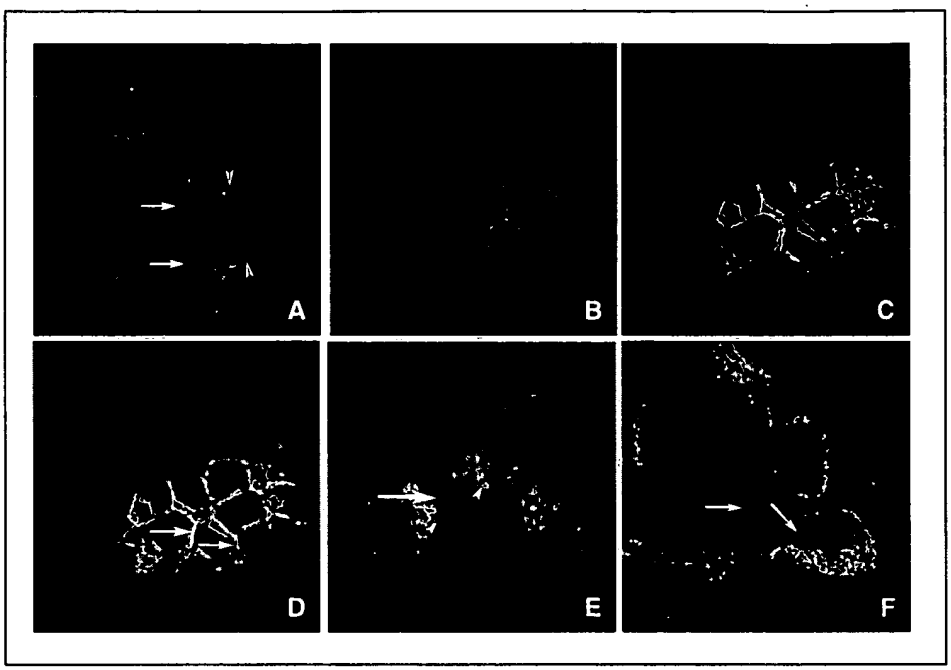

Figure 3. NGEP-L is present in the cell:cell contact regions in the LNCaP-CL-2 cells. *A*, immunofluorescence of NGEP-L was done on LNCaP-CL-2 using the rabbit NGEP-L antibody and the signal was detected using tetramethylrhodamine goat anti-rabbit. *Arrows*, NGEP-L (*red*) was predominantly localized in the cell:cell contact regions. There was also presence of NGEP-L in the plasma membrane (*arrowheads*) and also intracellular organelles. *B* to *D*, the LNCaP-CL-2 cells were stained with tetramethylrhodamine B isothiocyanate-labeled phalloidin (*B*; *red*) and the immunofluorescence of NGEP-L was done using rabbit NGEP-L antibody followed by Alexa 488–labeled goat anti-rabbit antibody (*C*; *green*). Phalloidin labels the F-actin beneath the plasma membrane and NGEP-L is present in the membrane of the cells. *Arrows*, NGEP-L is predominant in the cell:cell contact regions (*D*; *yellow*). *E*, LNCaP-CL-2 cells were stained with golgin-97 antibody, a *cis*-golgi protein (*green*), and costained with anti–NGEP-L antibody (*red*). *Arrowhead*, colocalization (*yellow*) of golgin-97 and NGEP; *arrow*, localization of NGEP in the cell-cell contact regions. *F*, LNCaP-CL-2 cells were stained with PDI antibody, an ER protein (*green*), and costained with anti-NGEP antibody (*red*). The nuclei were stained with DAPI.

pCDNA3.1-based vector as described in Materials and Methods. As shown in Fig. 2*C*, NGEP-L expression in Cl-2 (Fig. 2*C*, *lane 3*) is higher than expression in Cl-1 (Fig. 2*C*, *lane 2*). In addition, there is no detectable expression of NGEP-L in a vector-transfected LNCaP-V cell (Fig. 2*C*, *lane 1*). These cell lines were used for further experiments.

NGEP-L is a plasma membrane protein. To verify that NGEP-L was tightly associated with the membrane fraction of cells, LNCaP-CL-2 cells were disrupted by homogenization and subjected to low- and high-speed centrifugation, and the samples were analyzed by Western blots. As shown in Fig. 2*D*, NGEP-L was detected in both the low (Fig. 2*D*, *lane 2*) speed (1,000 × *g*) and the high (Fig. 2*D*, *lane 3*) speed membrane fractions (100,000 × *g*) but not in the soluble fraction of the cells (Fig. 2*D*, *lane 1*). In both membrane fractions (Fig. 2*D*, *lanes 2* and *3*), we observed a major band of the expected size (~ 100 kDa) for NGEP-L and a higher molecular weight band (probably an aggregate), which was not present in any of the fractions of the vector-transfected cells (Fig. 2*D*, *lanes 4–6*).

We next did immunofluorescence studies with LNCaP-CL-2 cells. F3 As shown in Fig. 3*A*, there is strong red fluorescence signal (tetramethylrhodamine) in the cell:cell contact regions and a weaker signal in the regions of the plasma membrane where the cells are not touching other cells. In addition, there is some punctate staining in the interior portion of the cell. To confirm the plasma membrane location of NGEP-L, we double stained cells using Alexa 488 (green) to stain NGEP-L and tetramethylrhodamine B isothiocyanate-labeled phalloidin to stain the F-actin network that lies very close to the plasma membrane. As shown in Fig. 3*B*, phalloidin stains the actin present adjacent to the plasma membrane region. Figure 3*C* shows the green NGEP-L signal that is very strong in cell:cell contact regions but also present inside the cell. Figure 3*D* shows that there is very good colocalization of NGEP-L and actin in the region of the cell membrane as would be expected at this level of resolution. The bright yellow signal at cell junction regions is consistent with an increase in NGEP-L in that region. There was no NGEP-L signal detected when we analyzed LNCaP-V cells or in LNCaP-CL-2 cells using prebleed sera establishing the specificity of the signal observed (data not shown).

To determine the location of NGEP-L protein within the cell, we did double labeling experiments with a *cis*-golgi marker (golgin-97) and with an ER marker (PDI) in LNCaP-CL-2 cell. As shown in Fig. 3*E*, there is colocalization of some of the intracellular NGEP-L signal with the *cis*-golgi marker but not with the ER marker (see Fig. 3*F*). It is likely that this signal represents NGEP-L being processed in the Golgi compartment before delivery to the cell membrane.

To determine the location of NGEP in normal prostate and prostate cancer, we did immunohistochemistry using four different formalin-fixed, paraffin-mounted prostate cancer specimens obtained from radical prostatectomy. Figure 4 shows the staining F4 of the NGEP in both normal and malignant glands from one of the prostate cancer specimens. In the normal glands, NGEP is present at the apical and the lateral intercellular region between acinar epithelial cells, when sectioned perpendicular to the basement membrane (Fig. 4*A*). Figure 4*B* shows the same expression pattern, when sectioned tangential to the basement membrane. Figure 4*C* and *D* shows the perpendicular and the tangential sections from

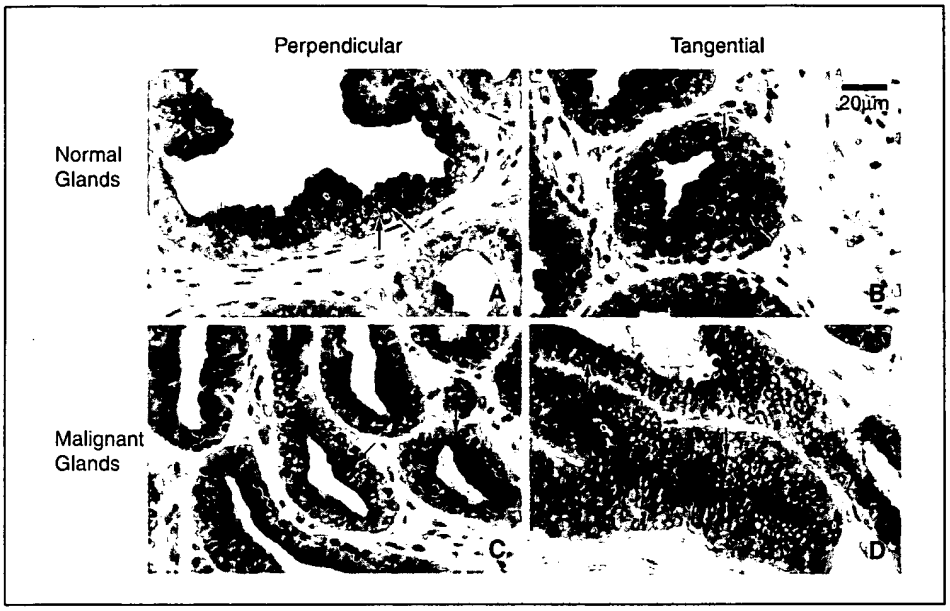

Figure 4. Location of NGEP-L in prostate. Slides were prepared from formalin fixed, paraffin-embedded human prostate cancer samples. NGEP-L was detected as described in Materials and Methods using a polyclonal antibody to NGEP-L at a dilution of 1:1,500. *Arrows*, NGEP-L signal on the lateral surface of the cells. *A* and *B*, normal prostate. *C* and *D*, prostate cancer. The signal is visible in both perpendicular and tangential sections of normal and malignant prostate glandular epithelia with respect to the basement membrane on which the epithelial cells sit. Perpendicular sections (*A* and *C*). Tangential sections (*B* and *D*).

malignant prostate adenocarcinoma glands, respectively. In the malignant prostate glands, NGEP was expressed on the lateral and the apical surface of the epithelial cells, similar to the distribution in normal prostate glands.

Effect of NGEP-L on cell morphology. To examine if expression of NGEP-L in the cell:cell contact regions can alter the behavior or shape of the LNCaP cells, we plated 4.5×10^4 cells from each of the two clones in a six-well plate and observed the cells for several days. Figure 5 shows phase-contrast micrographs of living cells taken on day 7. Two striking differences were observed when compared with untransfected LNCaP cells (Fig. 5*A*) or LNCaP-V cells (Fig. 5*B*). Cells expressing NGEP-L protein formed large aggregates and the cells not in aggregates were rounder and less spread out than control cells (Fig. 5*C* and *D*). This behavior was not due to clonal variation because we made the same observation in two different cell lines.

RNAi-mediated inhibition of NGEP-L expression. To determine if the aggregation and morphologic changes of the NGEP-L–expressing cells were due to expression of NGEP-L, we carried out siRNA experiments to lower the NGEP-L levels. We reasoned that if the change in the morphology of the cells is due to NGEP expression, then down-regulation of NGEP-L would revert the cell

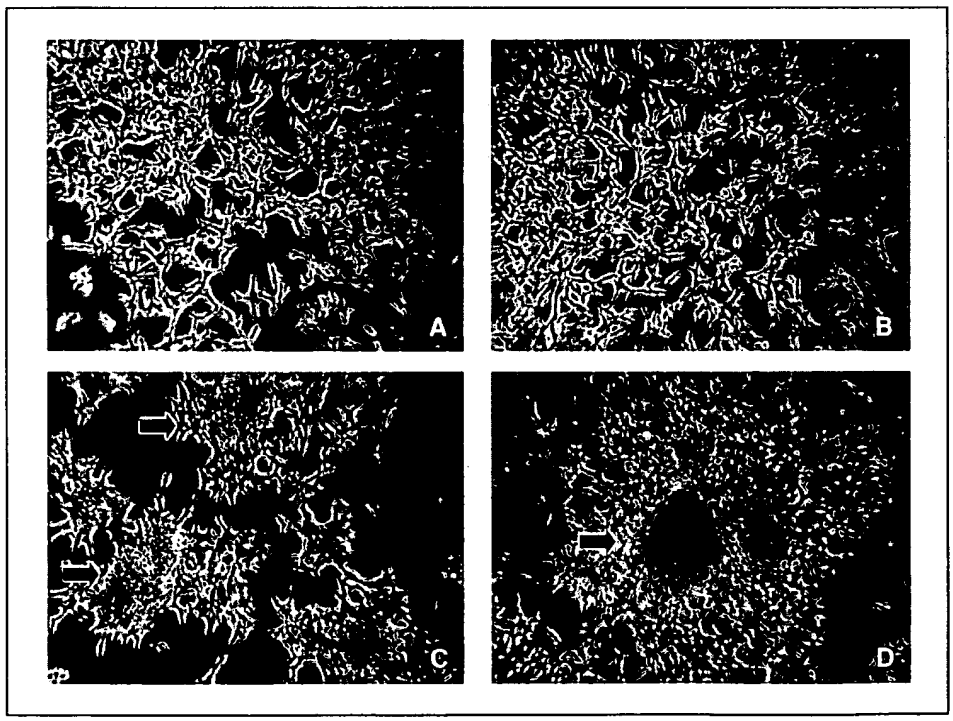

Figure 5. Expression of NGEP-L in the LNCaP cells alters the morphology of the cells. Phase-contrast photographs of growing LNCaP cells. *A*, parental LNCaP cells. *B*, LNCaP-V cells. *C*, LNCaP-CL-1. *D*, LNCaP-CL-2. NGEP expression shows a circular morphology, more cell:cell adhesion morphology compared with the control cells. *Arrows*, aggregation of the cells due to NGEP-L.

shape to that of the parental LNCaP cells and eliminate the aggregates. Four siRNA oligonucleotide sets were tested for their ability to lower NGEP-L levels measured on a Western blot before deciding on the best construct for the efficient knockdown. siRNA (D-023184-01) specific to NGEP-L was chosen and was used for the studies shown here. To show down-regulation of the NGEP-L protein level in the LNCaP CL-2 cells, Western blot analysis was F6 carried out. As shown in Fig. 6*A*, NGEP-L siRNA repressed the expression of NGEP-L protein in the LNCaP-CL-2 cells (Fig. 6*A*, *lanes 1–3*), but the control siRNA did not (Fig. 6*A*, *lane 4*). The maximum reduction in protein levels was observed 4 days after transfection.

To determine if the down-regulation of NGEP-L protein has any effect on the aggregation or morphology of the LNCaP-CL-2 cells, we monitored the cells after transfection with the siRNAs. Figure 6*B* to *G* shows the phase-contrast micrographs of the LNCaP-CL-2 cells transfected with NGEP-L–specific siRNA, control siRNA, and the nontransfected cells at days 4 and 6 after transfection. The LNCaP-CL-2 cells transfected with siRNA-NGEP-L show a morphology that is similar to that of the parental untransfected LNCaP cells (Fig. 6*B* and *E* and compare it with Fig. 5*A* and *B*). The cells are more elongated compared with the LNCaP cells expressing NGEP-L. The LNCaP-CL-2 cells (Fig. 6*D* and *G*) and the LNCaP-CL-2 cells transfected with nonspecific siRNA (Fig. 6*C* and *F*) have a similar morphology. They form clumps of cells as shown in Fig. 6*C*, *D*, *F*, and *G*, *arrows*.

To determine if the NGEP-L interaction responsible for the cell:cell adhesion requires calcium, we did Ca^{2+} chelation with EGTA. We reasoned if the cell:cell adhesion due to NGEP-L requires calcium, depletion of the extracellular calcium will cause the aggregates to come apart. We treated the LNCaP-CL-2 cells with 2 mmol/L EGTA for 1 h and monitored the cells. We found that EGTA treatment caused the LNCaP-CL-2 cells to detach from the dish. However, the few cells that remained on the plate were still in clumps (data not shown).

Discussion

The *NGEP* gene was originally identified by analyzing the EST database and searching for genes expressed in prostate and prostate cancer and not other tissues. Proteins encoded by such genes could be useful therapeutic targets for cancer therapy. An analysis of NGEP mRNA showed that there are two spliced variants (6). One of these encodes a short cytoplasmic protein (NGEP-S) and the other encodes a long polytopic membrane protein (NGEP-L), which is a member of the TMEM16 family of proteins (9, 11). The antibody used in the current study was generated against the COOH terminus of NGEP-L because that region is the most diverse among the family members. The antibody specifically detected a ~ 100-kDa protein in samples of normal prostate, prostate cancer, and BPH but not in other tissues examined, such as liver and brain. This finding indicates that the antibody is specific for NGEP-L. We then examined two prostate cancer cell lines, PC3 and LNCaP, for NGEP-L protein expression and it was not detected in either. This result was not surprising because NGEP mRNA is not detected in PC3 cells and is expressed at a very low level in LNCaP cells (6). We then used LNCaP cells to evaluate NGEP-L function because it is more differentiated than the PC3 line as evidenced by its production of the prostate-specific antigen and its response to androgen (12).

Two cell lines producing NGEP-L were established by transfecting a full-length NGEP-L cDNA into LNCaP cells. Western blots showed that these cell lines produced NGEP-L protein with the expected molecular weight and the levels were comparable with NGEP-L protein levels in prostate cancer specimens (Fig. 2*C*). Examination of the location of NGEP-L by confocal microscopy using the antibody to NGEP-L showed that NGEP-L was located in the plasma membrane and that its concentration was increased at cell contact regions. Furthermore, as the cells grew to high density, large aggregates of cells formed, suggesting that NGEP-L has an important role in promoting cell:cell interactions. These large

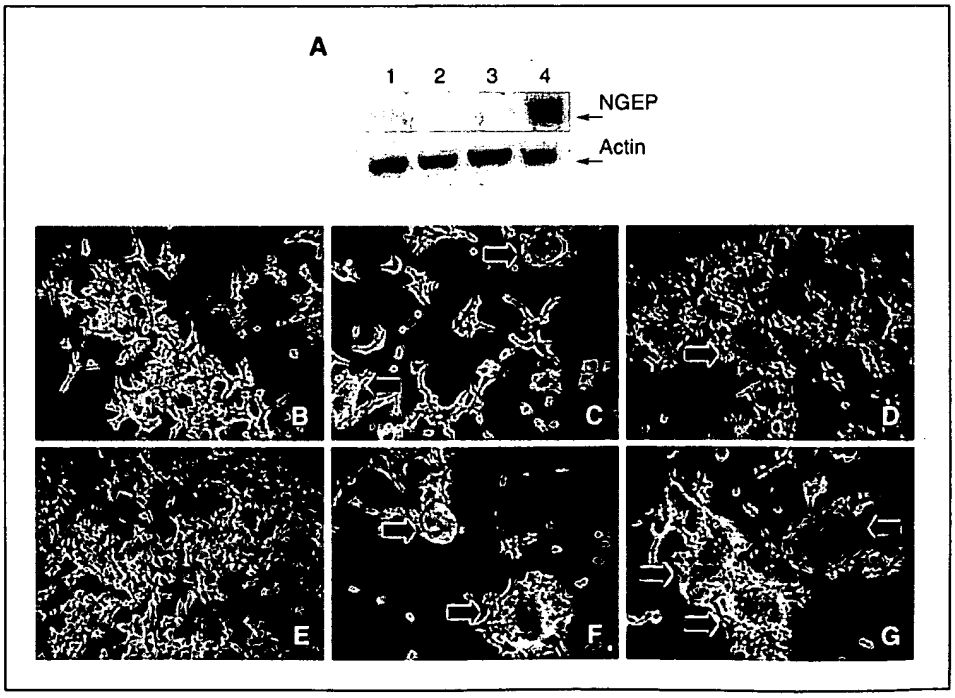

Figure 6. NGEP-L siRNA mediates gene silencing in LNCaP-CL-2 cells. *A*, Western blot analysis of the NGEP-L protein expression was done after 2, 4, and 6 d of transfection with RNA duplexes. A control siRNA (GL2-Luc) was used in parallel to test potential nonspecific effects of the short RNA duplexes. The unaffected actin expression was used as internal standard of protein concentration in each lane. Twenty micrograms of cell lysates after 2 d of siRNA-NGEP transfection (*lane 1*); 4 d of siRNA-NGEP transfection (*lane 2*); 6 d of siRNA-NGEP transfection (*lane 3*); and 6 d of siRNA-GL2-Luc transfection (*lane 4*) were loaded onto a gel and Western blot analysis was done using rabbit NGEP antibody (1:1,000). *B* to *G*, NGEP-L knockdown induces alteration in the morphology of the LNCaP-CL-2 cells. Phase-contrast photographs of growing LNCaP-CL-2 cells during *NGEP* gene silencing. LNCaP-CL-2 transfected with siRNA-NGEP at day 4 (*B*) and day 6 (*E*). LNCaP-CL-2 transfected with control siRNA (GL2-Luc) at day 4 (*C*) and day 6 (*F*). LNCaP-CL-2 cells at day 4 (*D*) and day 6 (*G*). *Arrows*, clump of cells present in the LNCaP-CL-2 and in the LNCaP-CL-2 cells transfected with nonspecific siRNA. The clumps disappeared in cells transfected with siRNA specific to NGEP-L.

aggregates were not observed in cells transfected with an empty vector or in nontransfected LNCaP cells. To confirm that aggregation was due to presence of NGEP-L protein, we showed that a RNAi for NGEP-L prevented the formation of large cellular aggregates.

We also examined the location of NGEP-L in both normal prostate and prostate cancers tissues using formalin fixed tissues obtained from prostatectomy samples. Figure 4 shows one example; the other three showed very similar results. In both normal and cancers, NGEP-L was localized on the apical and the lateral surfaces of the epithelial cells of prostate. This location is consistent with the finding with transfected LNCaP cells where accumulation of NGEP at cell contact regions was observed. The similar cell:cell contact location of NGEP-L in the LNCaP cells and in the prostate tissue suggests that NGEP-L may have an important role in the cell:cell interactions.

Connexins are gap junction proteins ubiquitously present in epithelial cells, including prostate, and have an important role in intercellular communication allowing the transfer of small molecules between cells. In many epithelial cancers, including prostate cancer, the levels of connexin are low or even absent (13–15). Connexin levels are low in LNCaP cells (16, 17) and overexpression of connexins has been shown to cause aggregation of LNCaP cells similar to that observed with NGEP-L–transfected LNCaP cells (16). However, unlike NGEP-L, connexins are not differentiation antigens with a specific role in the prostate. It is possible that NGEP-L, perhaps cooperating with connexins, has an important role in the formation of the prostate gland. We are currently making NGEP knockout mice to examine this hypothesis.

NGEP-L is a polytopic protein but its precise orientation of the protein in the membrane has not been established. NGEP-S, which shares a common NH_2 terminus with NGEP-L, is a cytosolic protein and it is likely that the NH_2 terminus of NGEP-L is also inside the cell (6). The COOH terminus of NGEP-L is probably also inside the cell because the antibodies described in this article that react with the COOH terminus only detect NGEP in permeabilized cells. We are currently producing mAbs to other regions of NGEP to learn more about its function and its orientation in the plasma membrane. mAbs to an extracellular portion of NGEP could be useful in the immunotherapy of prostate cancer.

Acknowledgments

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Generation of Human Cytotoxic T Cells Specific for Human Carcinoembryonic Antigen Epitopes From Patients Immunized With Recombinant Vaccinia-CEA Vaccine

*Kwong Y. Tsang, Sam Zaremba, Carol A. Nieroda, Ming Z. Zhu, J. Michael Hamilton, Jeffrey Schlom**

Background: **The human carcinoembryonic antigen (CEA), which is expressed in several cancer types, is a potential target for specific immunotherapy using recombinant vaccines. Previous studies have shown that when the CEA gene is placed into vaccinia virus, the recombinant vaccine (rV-CEA) can elicit T-cell responses in both rodents and nonhuman primates.** ***Purpose:*** **Our objective was to determine if rV-CEA could elicit CEA-specific T-cell responses in humans with appropriate human leukocyte antigen (HLA) motifs.** ***Methods:*** **Peripheral blood lymphocytes (PBLs) obtained from patients with metastatic carcinoma, both before and after vaccination with rV-CEA, were analyzed for T-cell response to specific 9- to 11-mer CEA peptides selected to conform to human HLA class I-A2 motifs.** ***Results:*** **While little or no T-cell growth was seen from preimmunization PBLs of patients pulsed with CEA peptides and interleukin 2 (IL-2), T-cell lines were obtained from PBLs of patients after vaccination with one to three cycles of stimulation. Cytolytic T-cell lines from three HLA-A2 patients were established with a 9-amino acid peptide (CAP-1), and the $CD8^+/CD4^+$ double-positive T-cell line (V24T) was chosen for detailed analysis. When autologous Epstein-Barr virus (EBV)-transformed B cells were either incubated with CAP-1 peptide or transduced with the CEA gene using a retroviral vector, they were lysed by the V24T cell line, but allogeneic non-A2 EBV-transformed B cells were not. The SW403 human colon carcinoma cell line, which is CEA positive and HLA-A2 positive, was also lysed by the V24T cell line, while two non–HLA-A2 CEA-positive colon carcinoma cell lines were not. To further confirm the class I HLA-A2 restricted nature of the V24T cytotoxicity, the non–HLA-A2 SW837 CEA-expressing colon carcinoma cell line was infected with a recombinant vaccinia virus expressing the HLA class I-A2 gene, and it became susceptible to V24T lysis. Cells infected with vector alone were not lysed.** ***Conclusions:*** **This study demonstrates for the first time (*a*) the ability to generate a human cytolytic T-cell response to specific epitopes of CEA, (*b*) the class I HLA-A2 restricted nature of the T-cell mediated lysis, and (*c*) the ability of human tumor cells to endogenously process CEA to present a specific CEA peptide in the context of major histocompatibility complex for T-cell–mediated lysis.** ***Implications:*** **These findings have implications in the development of specific second-generation cancer immunotherapy protocols. [J Natl Cancer Inst 87:982-990, 1995]**

The identification and selection of antigens and specific epitopes as targets for active immunotherapy approaches to human cancer are now in a dynamic phase. Specific peptides that bind human major histocompatibility complex (MHC) molecules have now been identified for melanoma-associated antigens (*1-4*). The identification of human carcinoma-associated antigens and epitopes that can be recognized by human T cells is also currently under active investigation. Molecules, such as prostate specific antigen (PSA) (*5,6*), c-erbB/2 (*7*), MUC-1 (*8*), point mutated ras (*9-11*), point mutated p53 (*12*), and carcinoembryonic antigen (CEA) (*13-15*) are among such candidates.

In humans, CEA is extensively expressed on the vast majority of colorectal, gastric, and pancreatic carcinomas as well as approximately 50% of breast cancers and 70% of non–small-cell lung cancers (*16*). CEA is also expressed, to some extent, on normal colon epithelium and in some fetal tissue (*16*). The CEA gene has been sequenced and shown to be part of the human immunoglobulin gene superfamily (*16,17*) and, thus, shares some homology with other molecules found on normal human tissues. At the amino acid level, CEA shares approximately 70% homology with nonspecific cross-reacting antigen (NCA), which is found on normal granulocytes (*16*).

The immunogenicity of CEA in humans is, at best, controversial. Several studies (*18,19*) claim antibodies to CEA in patients, while other investigators report these observations are artifacts (*20-22*). No reports of the presence or absence of human T-cell responses to CEA exist.

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See "Notes" section following "References."

One strategy that is being pursued to determine if T-cell responses to CEA can be induced in carcinoma patients is to place the CEA gene into vaccinia virus. Vaccinia was chosen as a vector for several reasons. Among these are (*a*) its wide use in humans in the eradication of smallpox; (*b*) its ability to infect a wide range of cells, including professional antigen-presenting cells (APCs), and express the product of an inserted gene such that it has the potential to be processed in the context of class I and/or class II MHC molecules; and (*c*) that animal model studies have shown that the use of a recombinant human CEA vaccinia virus (designated rV-CEA) is superior to the use of soluble CEA in the induction of antitumor effects on established CEA-expressing tumors (*13*). These findings correlated with the appearance of CEA-specific cytotoxic T-lymphocytes (CTLs) in rV-CEA–inoculated animals (*13*). rV-CEA also has been administered to rhesus monkeys and has been shown to induce CEA-specific T-cell responses with no toxicity (*14*).

It is important to emphasize, however, that experimental model results should have extremely limited extrapolation to potential human immune T-cell responses. Human CEA is a foreign gene in both mice and nonhuman primates. However, the more important point to consider is whether human APCs, including tumor cells, will process CEA in such a manner as to present specific CEA peptides in the context of human MHC for human T-cell recognition. Since mouse and nonhuman primate MHC-binding motifs are different from human motifs, studies in animal models cannot answer the question of T-cell immunogenicity in humans. Even the use of CEA-transgenic mice could not answer these questions, because they would possess murine MHC motifs. Thus, while animal model studies were conducted to demonstrate that rV-CEA can infect mammalian cells in vivo to such a level as to induce immune responses and to demonstrate the lack of toxicity, only clinical trials can adequately answer the question of the potential ability of rV-CEA to induce CEA-specific human T-cell responses.

A phase I clinical trial approved by the National Cancer Institute (NCI) Institutional Review Board and conducted by the NCI-Navy Oncology Branch involving the use of rV-CEA in 26 patients with metastatic carcinoma (gastrointestinal, lung, and breast) has recently been completed (*23*). No toxicity was observed other than that usually seen with the smallpox vaccine. A maximum tolerated dose was not achieved, even in the group that received rV-CEA injections of 10^7 plaque forming units (pfu) once a month for 3 months. While T-cell responses to vaccinia virus were observed (*23*), no primary T-cell lymphoproliferative response was observed when soluble CEA protein was presented to peripheral blood lymphocytes (PBLs) obtained prior to or after rV-CEA vaccination.

In an effort to further analyze CEA-specific T-cell responses as a result of rV-CEA vaccination, peptides reflecting potential human class I T-cell epitopes were selected and used with interleukin 2 (IL-2) to stimulate PBLs of patients before and after vaccination with rV-CEA. What has emerged is the first evidence for the induction of CEA-specific CTL responses in patients after rV-CEA vaccination.

Materials and Methods

rV-CEA

A recombinant vaccinia virus expressing CEA (rV-CEA) was generated by Therion Biologics Corporation (Cambridge, MA) using the methods described (*24*). The CEA gene was isolated as a complementary DNA (cDNA) clone from a human colon carcinoma cell cDNA library. The CEA cDNA was inserted, under the control of the vaccinia 40K promoter (*24*), into the *Hind* III M region of the genome of the attenuated strain of vaccinia virus (Wyeth strain).

Cell Cultures

Colorectal carcinoma cell lines SW403 (human leukocyte antigen [HLA]-A2 and HLA-A3), HT-29 (HLA-A1 and HLA-A9), SW837 (HLA-A19, -), and SW1417 (HLA-A3, -) were purchased from American Type Culture Collection (Rockville, MD). The cultures were mycoplasma free and were maintained in complete medium (Dulbecco's modified Eagle medium; Life Technologies, Inc. [GIBCO BRL], Grand Island, NY) supplemented with 10% fetal bovine serum (FBS), 2 m*M* glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin (Life Technologies, Inc.). The T2 cell line (transport deletion mutant) (*25*) was provided by Dr. Peter Cresswell (Yale University School of Medicine, New Haven, CT) and was maintained in Iscove's modified Dulbecco's medium (IMDM) containing 10% FBS.

Epstein-Barr virus (EBV)-transformed B cell lines designated as B-Vac24 and B-Vac01, and the B-Vac24 transfected with a retroviral vector containing the CEA gene [designated as B-Vac24(CEA)] were maintained in RPMI-1640 medium supplemented with 10% pooled human AB serum (Pel Freeze Clinical System, Brown Deer, WI), 2 m*M* glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin (Life Technologies, Inc.).

Peptide Synthesis

The peptide sequence of CEA was scanned for matches to the consensus motifs for HLA-A2 and HLA-A3 binding peptides. HLA-A2 and HLA-A3 alleles were chosen, since they are the most commonly expressed class I alleles. Peptides (9-, 10- and 11-mers) were selected for synthesis if they (*a*) conformed to the respective consensus motifs and (*b*) diverged sufficiently from NCA and human biliary glycoprotein (BGP), so that a response to these antigens would not be anticipated. A peptide that corresponded to the CAP-1 peptide (*see* below) after optimal alignment with NCA and CEA was also synthesized and designated NCA-1. Syntheses were performed on a peptide synthesizer (model 432A; Applied Biosystems, Foster City, CA), and products were dissolved in aqueous solution, sterile filtered, and frozen at −70 °C at a concentration of 2 mg/mL. The purity of the peptides was greater than 90% as analyzed by high-performance liquid chromatography (HPLC). The CEA peptides are listed in Table 1.

Introduction of the CEA cDNA in EBV-Immortalized B-Cell Lines

Since tumors from rV-CEA vaccinated patients were not available, EBV-transformed B cells from these patients were used as autologous targets for T cells. B-cell lines were generated by a standard method (*26*) using B95-8 marmoset cell line supernatant containing EBV. Human pooled AB serum was used in all cell cultures in this study. EBV-immortalized B-cell lines were transduced with a retroviral expression construct of CEA (*27*). Transduction was performed by cocultivation of EBV-immortalized B cells with productively transduced amphotropic retroviral packaging cell line PA317-CEA as described by Tsang et al. (*28*). EBV-immortalized B-cell transductants were selected in medium containing G418 (geneticin) at an active concentration of 0.7 mg/mL. The purpose of the generation of immortalized cells was to have a continuous supply of targets to analyze cytotoxic T-cell responses.

Generation of T-Cell Lines

Peripheral blood mononuclear cells (PBMCs) were obtained from heparinized blood from patients with metastatic carcinoma who were enrolled in a phase I trial employing rV-CEA (*13,14*). All experiments involving patient materials were conducted according to NIH guidelines and written, informed consent was obtained from all patients. PBMCs were obtained prior to and after administration of injections of rV-CEA given once a month for 3 months at 2 ×

Table 1. Binding of CEA peptides to the HLA class I-A2 molecule

Peptide	Amino acid position in CEA	Sequence	Predicted binding to HLA class I-A2*	T2 binding assay†	
				Experiment 1	Experiment 2
CAP-1	571-579	YLSGANLNL	P	561	806
CAP-2	555-564	VLYGPDTPII	P	515	796
CAP-3	87-96	TLHVIKSDLV	P	480	ND
CAP-4	1-11	KLTIESTPFNV	P	441	ND
CAP-5	345-354	TLLSVTRNDV	P	405	ND
CAP-6	19-28	LLVHNLPQHL	P	381	ND
CAP-7	27-35	HLFGYSWYK	N	326	ND
CAP-8	523-532	TLFNVTRNDA	N	260	ND
CAP-9	137-146	TQDATYLWWV	N	204	ND
CAP-10	102-110	GQFRVYPEL	N	201	ND
NCA-1	571-579	YRPGENLNL	N	252	225
Positive control	—	ALAAAAAAV	P	632	—
No peptide	—	—	—	280	300

*Predicted binding on the basis of published motifs (*31*); P = positive; N = negative.

†Reactivity of T2 cells with anti–HLA-A2 MAb after the cells were incubated with CEA peptide. Peptides were used in a concentration of 50 μg/mL/10^6 cells. The results are expressed in relative fluorescence values (350 was arbitrarily chosen as a cutoff value for positive). Positive control 9-mer is an A2-binding motif.

10^5 pfu (patient Vac7), 2×10^6 pfu (patient Vac6), and 10^7 pfu (patients Vac24, Vac23, and Vac32) per injection. PBMCs from patients were separated using lymphocyte separation medium gradient (Organon Teknika, Durham, NC) as previously described (*29*). Washed PBMCs were resuspended in complete medium: RPMI-1640 (Life Technologies, Inc.) supplemented with 10% pooled human AB serum (Pel Freeze Clinical System), 2 m*M* glutamine, 100 U/mL penicillin, and 100 μg/mL of streptomycin (Life Technologies, Inc.). Cells (2×10^5) in complete medium in a volume of 100 μL were added into each well of a 96-well flat-bottom assay plate (Corning Costar Corp., Cambridge, MA). Peptides were added to cultures at a final concentration of 50 μg/mL. Cultures were incubated for 5 days at 37 °C in a humidified atmosphere containing 5% CO_2. After removal of the peptide-containing medium, the cultures were then provided with human IL-2 (provided by the NCI Surgery Branch) (10 U/mL) for 11 days, with IL-2–containing medium being replenished every 3 days. The incubation time of 5 days with peptide plus 11 days with IL-2 constitutes one cycle. Primary cultures were restimulated with the same peptide (50 μg/mL) on day 16 to begin the next cycle. Irradiated (4000 rad) autologous peripheral blood mononuclear cells (5×10^5) were added in a volume of 50 μL in complete medium as APCs. T-cell lines derived from patients Vac24, Vac6, Vac7, etc. were given the designations V24T, V6T, V7T, etc., respectively.

Cytotoxicity Assays

Various target cells were labeled with 50 μCi of ^{111}In-oxyquinoline (Medi-Physics Inc., Arlington, IL) for 15 minutes at room temperature. Target cells (0.5×10^4) in 100 μL of complete medium (see below) were added to each of 96 wells in U-bottom assay plates (Corning Costar Corp.). The labeled targets were incubated with peptides at a final concentration of 50 μg/mL for 60 minutes at 37 °C in CO_2 before adding effector cells. Effector cells were suspended in 100 μL of complete medium supplemented with 10% pooled human AB serum and added to target cells; the plates were then incubated at 37 °C in 5% CO_2 for 12 or 18 hours. Supernatant was harvested for gamma counting with the use of harvester frames (Skatron, Inc., Sterling, VA). Determinations were carried out in triplicate and standard deviations were calculated. All experiments were carried out three times, except for data in Table 7, which was done twice. Specific lysis was calculated with the use of the following formula:

$$\% \text{ lysis} = \frac{\text{observed release (cpm)} - \text{spontaneous release (cpm)}}{\text{total release (cpm)} - \text{spontaneous release (cpm)}} \times 100.$$

Spontaneous release was determined from wells to which 100 μL of complete medium was added. Total releasable radioactivity was obtained after treatment of targets with 2.5% Triton X-100.

Detection of Tumor Necrosis Factor-α

Supernatants of T cells exposed for 3 days to peptides and APC in IL-2–free medium, at a responder to stimulator ratio of 4:1 (4×10^6:1 $\times 10^6$ cells/mL), were screened for the secretion of tumor necrosis factor-α (TNF-α), using an enzyme-linked immunosorbent assay kit (Genzyme Corp., Cambridge, MA). The results were expressed in pg/mL.

Flow Cytometry

The procedure for single-color flow cytometric analysis has been previously described (*30*). Briefly, 1×10^6 cells were washed three times with cold Ca^{2+}- and Mg^{2+}-free Dulbecco's phosphate-buffered saline (DPBS) and then stained for 1 hour with 1 μg of monoclonal antibody (MAb) against CD3 (Becton Dickinson, San Jose, CA), CD4 (Becton Dickinson), CD8 (Becton Dickinson), HLA class I (W6/32) (Sera-Lab, Sussex, England), HLA class II (HLA-DR) (Becton Dickinson), and MOPC-21 (Cappel/Organon Teknika Corp., West Chester, PA) in a volume of 100 μL of PBS containing 1% bovine serum albumin. Anti-CEA MAb COL-1 was used as 100 μL of culture supernatant. The cells were then washed three times with cold DPBS and incubated for an additional hour in the presence of 1:100 dilution (volume of 100 μL PBS containing 1% bovine serum albumin) of fluorescein-conjugated goat anti-mouse immunoglobulin (Ig) (Kirkegaard & Perry Labs., Gaithersburg, MD). The cells were again washed three times with DPBS and resuspended in DPBS at a concentration of 1×10^6 cells/mL. The cells were immediately analyzed using a Becton Dickinson FACScan equipped with a blue laser with an excitation of 15 nW at 488 nm. Data were gathered from 10 000 live cells, stored and used to generate results.

The procedure for dual-color flow cytometric analysis was similar to that for single-color analysis, with the following exceptions. The antibodies used were anti-CD4 fluorescein conjugate, anti-CD8 phycoerythrin conjugate, anti-IgG_1 fluorescein conjugate, and anti-IgG_{2a} phycoerythrin conjugate (Becton Dickinson). Staining was done simultaneously for 1 hour after which cells were washed three times, resuspended as above, and immediately analyzed using a Becton Dickinson FACSort equipped with a blue laser with an excitation of 15 nW at 488 nm equipped with the Lysis II program.

Binding of CEA peptides to the HLA-A2 molecule was analyzed by the up-regulation of HLA-A2 expression on T2 cells (*25*) as demonstrated by flow cytometry. The T2 cell peptide binding assay has been reported previously (*31*). Briefly, aliquots of $0.5\text{-}1 \times 10^6$ T2 cells in serum-free IMDM were incubated with peptides at a concentration of 50 μg/mL in 24-well culture plates at 37 °C in 5% CO_2 overnight. Flow cytometry for peptide binding was carried out using T2 cells and single-color analysis. After cells were washed three times in DPBS as above, they were incubated for 1 hour with HLA-A2 specific antibody A2,28 (#189HA-1; One Lambda, Inc., Canoga Park, CA), using 10 μL of a 1× working dilution/10^6 cells. MOPC-104E (Cappel/Organon Teknika Corp., West Chester, PA) was used as isotype control. The cells were then washed three times and incubated with a 1:100 dilution of phycoprobe PE (phycoerythrin) labeled anti-mouse IgM (Biomeda Corp., Foster City, CA). Analysis was carried out using the FACScan as described above. Cells were maintained on ice during all cell preparation and staining unless otherwise stated above.

HLA Typing

The HLA phenotyping of patients was performed by the Tissue Typing QC Laboratory, Naval Medical Research Institute, Bethesda, MD, using a standard antibody-dependent microcytotoxicity assay and a defined panel of anti-HLA antisera. The HLA phenotypes were as follows: patient Vac24 (HLA-A2, 24; B 44, 51; DR 4, 11; DQ 3, 7; DR w52, 53); patient Vac01 (HLA-A28, 31; B14, 35; DR1, 4; DQ1, 3; DRw53); patient Vac23 (HLA-A1, 26; B8, 60; CW3, 7; DR0103, 15; DQ5, 6); patient Vac32 (HLA-A3, 68; B7, 51; CW7; DR4, 15; DQ1, 8; DRw53); patient Vac6 (HLA-A2, 24; B13, 51; CW6; DR7, 8; DQ4; DR53); and patient Vac7 (HLA-A2; B7; CW7; DR15, 17; DQ1, 2; DR52). From patient Vac01, only B cells were used.

Vaccinia Virus Infection of Colorectal Carcinoma Cells

cDNA for the HLA-A2.1 gene in the vaccinia virus vector was provided by the Surgery Branch, NCI, National Institutes of Health. These genes were inserted into the TK gene in plasmid pSCII, allowing homologous recombination to occur with the viral TK gene (*32*). Target cells at a concentration of 1×10^7/mL in complete RPMI-1640 medium supplemented with 0.1% bovine serum albumin were incubated with an equal volume of vaccinia virus (10^8 pfu/mL) in the same medium at 37 °C for 1.5 hours. The cells were then adjusted to a concentration of 5×10^5/mL in complete medium and incubated for 3 hours at 37 °C.

Statistical Analysis

Statistical analysis of differences between means was done by a two-tailed paired *t* test.

Results

Identification of Potential CEA-Specific T-Cell Epitopes

Since the entire amino acid sequence of human CEA is known and human HLA class I-A2 consensus motifs have been described (*33,34*), studies were undertaken to identify a series of peptides that would potentially bind class I-A2 molecules. A2 was chosen, since it is the most common HLA class I molecule, being represented in approximately 50% of North American caucasians and 34% of African-Americans (*35*). The peptide sequence of CEA was thus examined for matches to the consensus motifs for HLA-A2 binding peptides. Peptides were only selected, moreover, if their sequence diverged sufficiently from the CEA-related NCA and BGP sequences. The amino acid sequence of human CEA (GeneBank Accession #M17303) was scanned using a predictive algorithm (*36*) that combines a search for anchor residues with numerical assignments to all residues at all positions. Ten peptides were synthesized using this algorithm, ranging in length from 9 to 11 amino acids. Six of these peptides also contained the HLA-A2 binding motif of leucine or isoleucine at position 2 and valine or leucine at the C terminal. Another peptide (CAP-7) also possessed the motif for binding to HLA-A3 (*37*). All peptides were selected to have minimal homology to the parallel regions of NCA and BGP after optimal alignment of the latter sequences with CEA. The 9-mer, 10-mer, or 11-mer peptides that met these criteria were selected for synthesis and purification; they were designated CAP (carcinoembryonic antigen peptide)-1 through 10. Their amino acid sequence and position in the CEA molecule are given in Table 1. The positive (P) or negative (N) designation (Table 1) relates to the predicted binding to HLA-A2.

The T2 cell-binding assay has been used to predict human HLA-A2 consensus motifs (*31*). In this assay, the binding of an appropriate peptide results in the up-regulation of surface HLA-A2 on the T2 cells, which can be quantified via FACScan using an anti–HLA-A2 antibody. As seen in Table 1, six of the CEA peptides (CAP-1 through CAP-6) scored positive for T2 binding (the peptides were designated CAP-1 through CAP-10 retrospectively on the basis of their quantitative binding to T2). In general, the peptides with the HLA-A2 consensus motif were better binders than those lacking the motif. The order of T2 cell-peptide binding did not always correspond to the predictive algorithm (*36*). Since peptide 571-579 (designated CAP-1) demonstrated the highest level of T2 binding, the peptide reflecting the NCA analog (the corresponding NCA peptide obtained after optimal alignment of NCA and CEA) was also synthesized and tested; this peptide, designated NCA-1, showed background binding to T2 cells (Table 1). This low level of binding was consistent with the fact that an amino acid substitution in NCA had abolished one of the A2 anchor residues (Arg for Leu at position 2).

Establishment of T-Cell Lines to CEA Peptides

In an attempt to establish T-cell lines from patients who had received the rV-CEA construct, PBLs were obtained from three patients (designated Vac6, Vac7, and Vac24) with the HLA-A2 allele and were alternately pulsed with 50 μg/mL peptide CAP-1 and IL-2 (10 U/mL) as described in the "Materials and Methods" section. In all three cases, T-cell lines could be established that were cytotoxic for T2 cells when pulsed with the CAP-1 peptide. Fig. 1 shows the results of these assays using T-cell lines from patients Vac24 and Vac6. The T-cell line from patient Vac24 was chosen for further study.

PBLs from patient Vac24 (before and after vaccination with three doses of 10^7 pfu rV-CEA at monthly intervals) were placed in 96-well plates and pulsed with the CAP-1 peptide and then IL-2, as described in the "Materials and Methods" section. Each exposure to peptide and IL-2 was considered one cycle of stimulation. As seen in Table 2, one, two, or three cycles of CAP-1 peptide and IL-2 did not result in growth of cells in any of the 96 wells using the preimmunization PBLs. By contrast, after one cycle of stimulation of post-vaccination PBLs from the same patient, 66 of the 96 wells (69%) demonstrated growth of cells, which were maintained through four cycles of stimulation. It is of interest that, after four cycles of stimulation of preimmunization PBLs, two (2%) of 96 wells exhibited cell growth. Thus, one could hypothesize that a minor population of T cells exists in this patient that is capable of recognizing a specific CEA epitope (peptide 571-579) and that these cells were clonally expanded as a result of the rV-CEA administration.

Sufficient PBLs, before and after vaccination with rV-CEA at the 10^7 pfu dose, were also available from two non–HLA-A2 patients: Vac32 (HLA A1,26) and Vac23 (HLA A3,68). Since we had little or no basis for predicting which peptides might bind to these haplotypes, nine of the CEA peptides were used in an attempt to establish T-cell lines. Using peptide CAP-1 with IL-2 as described above, no T-cell lines could be established from preimmunization PBLs from either patient Vac32 or Vac23 (Table 2). However, employing post–rV-CEA immunization PBLs, T-cell lines were established after three cycles of stimulation in 25 (52%) of 48 wells for patient Vac32 and in 21 (44%) of 48 wells for patient Vac23 (Table 2).

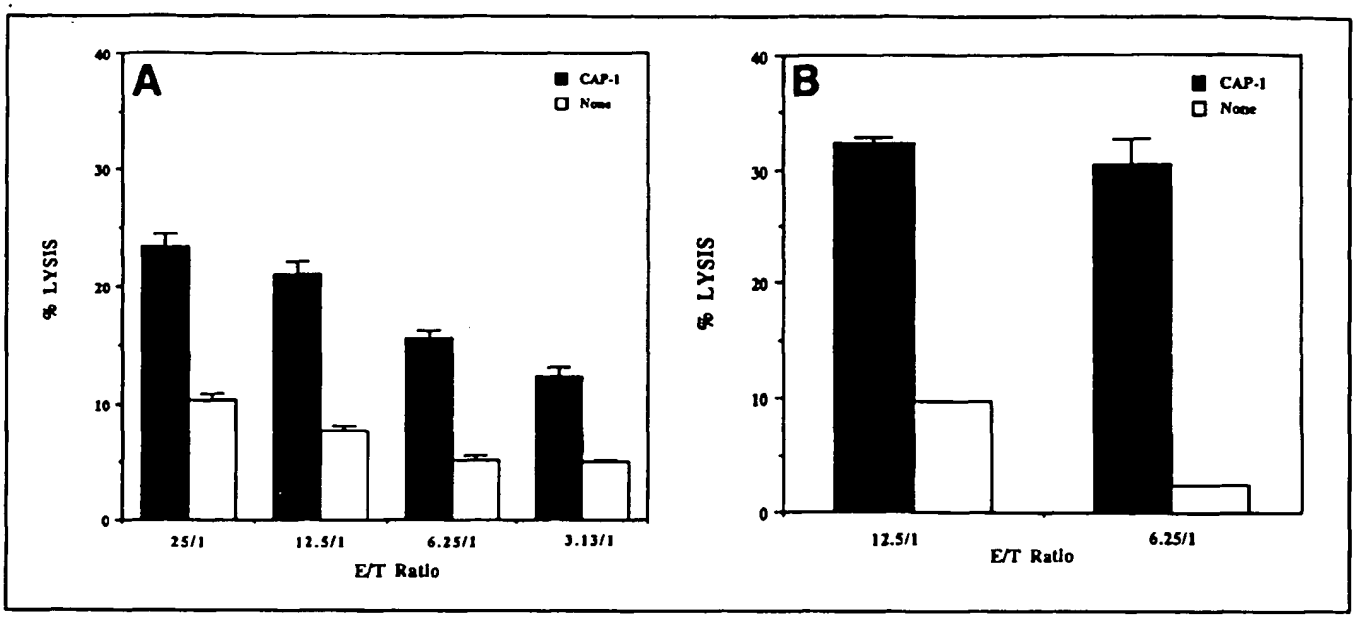

Fig. 1. Cytotoxicity of T-cell lines (designated V24T [Panel A] and V6T [Panel B]) derived from patients immunized with rV-CEA and induced by CEA CAP-1 peptide. CTL activity was determined in an 18-hour ^{111}In release assay using T2 cells as a target incubated with CAP-1 peptide (50 μg/mL). E/T ratio = effector-to-target ratio.

A similar contrast in pre-vaccination versus post-vaccination PBLs from patients Vac32 and Vac23 was seen with a mixture of CEA peptides CAP-4, CAP-6, and CAP-7 (Table 2). Combinations of peptides were used to conserve PBLs. It is of interest that PBL from patient Vac32 (HLA-A3 positive) showed evidence of cell growth in the presence of CAP-7, the peptide that bears the HLA-A3–binding motif. It should be noted that these results suggest that a peptide shown to bind to HLA-A2 can also stimulate T-cell lines after binding to some non-A2 antigens; the possible reasons for this will be discussed in detail below. However, it was decided to first characterize the T-cell responses in patient Vac24 because of the implied relevance of MHC binding and T-cell activation. Nonetheless, it is encouraging that PBLs from five of five patients showed signs of T-cell response to peptide CAP-1 after immunization with rV-CEA.

Flow Cytometry Analysis

Flow cytometric studies were conducted to phenotype the V24T cell line and V6T and V7T cells obtained by pooling respective cells growing in 96-well plates. The results are shown in Table 3. Cells stained double positive for both CD8 and CD4 in V24T and V6T cell lines, while the V7T cell line was CD8 positive.

Cytotoxicity Assays

To determine if the T-cell line from patient Vac24 (designated V24T) could lyse autologous B cells presenting the CAP-1 peptide, B cells from this patient were first transformed with EBV and then pulsed (i.e., incubated) with the CAP-1 peptide. As seen in Table 4, the V24T cells were capable of lysing the

Table 2. Frequency of T-cell growth following an in vitro stimulation with CEA peptides: before and after immunization with rV-CEA

		Frequency							
		Before immunization*				After immunization*			
Patient	Peptide	1	2	3	4	1	2	3	4
Vac24†	CAP-1	0	0	0	2	66	66	66	66
Vac32‡	CAP-4, CAP-6, CAP-7	0	0	4	ND	0	0	30	ND
	CAP-3, CAP-10, CAP-9	0	0	0	ND	0	0	0	ND
	CAP-8, CAP-2	0	0	0	ND	0	0	0	ND
	CAP-1	0	0	0	ND	0	0	25	ND
Vac23‡	CAP-4, CAP-6, CAP-7	0	0	3	ND	0	0	31	ND
	CAP-3, CAP-10, CAP-9	0	0	0	ND	0	0	0	ND
	CAP-8, CAP-2	0	0	0	ND	0	0	0	ND
	CAP-1	0	0	0	ND	0	0	21	ND

*Cycle of stimulation with peptide and IL-2 (*see* "Materials and Methods" section); ND = not done.

†Results are expressed as the number of positive wells per 96 wells. Peripheral blood mononuclear cells were seeded at a concentration of 1×10^5 cells per 200 μL/well.

‡Results are expressed as the number of positive wells per 48 wells. Peripheral blood mononuclear cells were seeded at a concentration of 1×10^6 cells per well in 1 mL.

Table 3. Flow cytometric analysis of surface markers on T-cell lines*

Surface antigen†	Cell line V24T	V6T	V7T
$CD8^+/CD4^+$	62.4	30.8	Negative
$CD8^+/CD4^-$	35.7	56.0	99.6
$CD4^+/CD8^-$	Negative	12.6	Negative
$CD3^+$	98.5	95.4	99.4

*Negative = less than 5% positive. Results are expressed in percentage of each T-cell line reactive with the MAbs. Routinely, 2%–4% of the cells were stained when treated either with no primary MAb or an isotype-matched control MAb.
†+ = positive; – = negative.

Table 4. Ability of V24T cells (T cells derived from PBLs from rV-CEA–vaccinated patient Vac24) to lyse autologous and allogeneic B cells pulsed with a CEA-specific peptide*

B cells	HLA-A2†	Pulsing peptide	% lysis (± SD)
B-Vac24	Positive	CAP-1	43 (0.29)†
		NCA-1	8 (1.92)
		None	2 (0.37)
B-Vac01	Negative	CAP-1	10 (0.53)
		NCA-1	11 (0.62)†
		None	8 (0.68)

*An 18-hour ^{111}In release assay was performed. Peptides were used in a concentration of 50 μg/mL. Results are expressed as percentage specific lysis at effector-to-target ratios of 25:1.
†Statistically significant ($P<.01$, paired t test). Similar statistically significant lysis was observed at effector-to-target ratios of 12.5:1.

autologous B cells when pulsed with CAP-1, but when an allogeneic (non–HLA-A2) EBV-transformed B cell was pulsed with the same peptide, no lysis was observed. Lysis was observed at effector-to-target cell ratios of 25:1 and 12.5:1. When the NCA-1 peptide, reflecting the analogous region on the NCA molecule was used to pulse B cells of patient Vac24, no lysis was observed with the V24T cells. As shown in Table 1, this lack of lysis was not unexpected, since three of the nine amino acids of NCA-1 differ from those of CAP-1, including an anchor residue.

Studies were then undertaken to determine if the CAP-1 peptide could induce the secretion of TNF-α from the cytolytic V24T cells. Incubation of V24T cells with autologous B cells pulsed with CAP-1 peptide resulted in the production of more than 300 pg/mL of TNF-α, while incubation with control or non-T2 cell-binding peptides CAP-9 and CAP-10 or no peptide showed levels of production below 75 pg/mL.

Cytotoxicity of V24T Cells Against Tumor Cells

While the above studies indicate that autologous B cells can present the CAP-1 peptide to the V24T cells, resulting in lysis of the B cells, they do not indicate that human APCs can endogenously process the entire CEA molecule in a manner so as to bind HLA-A2 molecules for presentation at the cell surface. To help clarify this issue, EBV-transformed B cells of patient Vac24 were transduced with the entire human CEA gene, using a retroviral vector (*see* "Materials and Methods" section). As seen in Table 5, the CEA-transduced cells now express CEA, and the transduction process had no effect on the expression of HLA class I and class II molecules.

Table 5. Flow cytometric analysis of surface antigens of EBV-transformed B cells derived from patient Vac24 before and after transfection with CEA

Antigen	MAb	% positive* B-Vac24†	B-Vac24 (CEA)‡
CEA	COL-1	4.4 (24.7)	42.9 (100.9)
HLA class I	W6/32	100.0 (831.9)	100.0 (519.1)
HLA class II	anti–HLA-DR	99.8 (313.3)	99.3 (221.8)
Control	MOPC-21	2.2 (30.5)	2.0 (24.1)

*Values represent the percentage of each cell type reactive with MAbs listed as analyzed by flow cytometry. Numbers in parentheses are the mean channel fluorescence intensity as determined in relative log units. Routinely, 2%–4% of the cells were stained when treated either with no primary MAb or an isotype-matched control MAb. LS-174T, a colorectal carcinoma cell line, was used as a positive control for CEA expression. The percent positive value for CEA in CEA-expressing LS-174T human colon carcinoma cell line was 59.8 (144.4).
†B-Vac24 are EBV-transformed B cells derived from PBLs of patient Vac24 prior to immunization with rV-CEA.
‡B-Vac24(CEA) are the same as B-Vac24 except they have been transduced with the entire human CEA gene using a retroviral vector as described in the "Materials and Methods" section.

As shown in Table 6, the autologous B cells transduced with the CEA gene can now serve as targets for the V24 CTLs. These results thus demonstrate that a CEA gene product can be endogenously processed by autologous B cells and presented at the cell surface in the context with class I MHC to induce T-cell lysis. The question now remained as to whether human carcinoma cells can act in the same manner as APCs and, thus, serve as potential targets for V24T cells. As seen in Table 6, non-A2 allogeneic carcinoma cells SW1417 and HT-29, which do express substantial CEA, cannot serve as targets, while the allogeneic A2-positive SW403 carcinoma cells expressing CEA are lysed at effector-to-target ratios of 50:1 and 25:1.

Cytotoxicity of V24T Cells Against Vaccinia-CEA–Infected Tumor Cells

To further demonstrate the HLA-A2 restricted nature of the V24T cells in the lysis of human carcinoma cells, the CEA-positive, non–HLA-A2 SW837 human carcinoma cell line was employed. These cells were either uninfected, infected with wild-type vaccinia virus, or infected with a recombinant vaccinia virus containing the HLA-A2 gene. Twelve-hour lysis ex-

Table 6. Cytotoxicity of V24T cell line (derived from patient Vac24 immunized with rV-CEA) on target cells with endogenous CEA expression*

Target	HLA-A2	CEA	% lysis (± SD)
B-Vac24†	Positive	Negative	8.2 (2.1)
B-Vac24 (CEA)†	Positive	Positive	46.1 (11.6)§
SW403‡	Positive	Positive	45.2 (1.5)§
SW1417‡	Negative	Positive	5.2 (0.5)
HT-29‡	Negative	Positive	4.1 (0.6)

*HLA-A2 and CEA expression were tested by flow cytometry using MAbs anti-A2 and COL-1, respectively. An 18-hour ^{111}In release assay was performed. Results are expressed in percent specific lysis at effector-to-target ratio of 50:1 compared with lysis obtained with B-Vac24 cells. Similar statistically significant lysis was seen at effector-to-target ratio of 25:1.
†As described in the legend to Table 4.
‡Human colon carcinoma cell lines expressing CEA.
§Statistically significant lysis ($P<.01$, paired t test).

periments were carried out to avoid spontaneous lysis due to vaccinia virus. As seen in Table 7, only the carcinoma cells infected with the rV-A2.1 recombinant expressing HLA-A2 were susceptible to lysis with V24T cells. These studies further demonstrate the HLA-A2 restricted nature of the CEA-specific lysis of the V24T cells.

Discussion

These studies demonstrate that one can evoke a cytotoxic T-cell response to a specific epitope of the human CEA molecule by vaccination with rV-CEA. This response appears to be mediated via a class I MHC restricted mechanism. Moreover, the lytic T cells generated against the defined CEA peptide were able to lyse tumor cells endogenously synthesizing the entire CEA antigen. The ability to raise a CTL response versus CEA in humans was clearly not a foregone conclusion prior to these studies; since CEA is expressed in fetal tissue and some normal colonic mucosa, "tolerance" to this molecule was one possible outcome. Moreover, previous studies dealing with the presence of antibodies to CEA were inconclusive and contradictory (*38-41*), and no studies have reported either the presence or the absence of cytolytic T-cell responses to CEA in humans. It was for this reason that the CEA gene was placed into vaccinia virus. A previous study (*13*) in a mouse model demonstrated enhanced CTL responses when employing rV-CEA as an immunogen as opposed to native CEA.

Other data are currently being accumulated on the potential immunogenicity of CEA. While the patients in the study reported here had advanced metastatic disease, a trial using the same rV-CEA construct described here, at the same dose schedule and route of administration, is currently under way in gastrointestinal cancer patients with minimal disease. In those studies, primary lymphoproliferative T-cell responses to CEA have been observed (*42*). In another study (*43*), an anti-idiotype MAb to an anti-CEA MAb has been administered to gastrointestinal cancer patients; in that study, antibodies to CEA and lymphoproliferative responses to CEA were reported. It should also be noted that a pilot phase I study (*44*) was previously carried out in which soluble CEA was administered to patients with advanced gastrointestinal carcinomas. In that study, as well as the other studies cited, no toxicity was observed. The findings of these phase I trials thus indicate that CEA deserves further evaluation as a potential target for immunotherapeutic applications.

Table 7. Demonstration of HLA-A2 involvement in ability of V24T cells to lyse human colon carcinoma cells*

Infection	Expression of MHC class I molecules†		% lysis (± SD)‡
	W6/32 antibody	A2,28 antibody	
None	99.1 (228.23)	4.4 (47.41)	8.2 (1.4)
Vaccinia A2.1	99.3 (261.74)	90.4 (66.70)	31.4 (2.5)§
Vaccinia vector control	99.4 (217.59)	2.9 (17.54)	7.2 (0.6)

*SW837, an HLA-A2–negative and CEA-positive human colorectal carcinoma cell line, was infected with vaccinia A2.1 vector. The surface expression of HLA-A2 molecule after infection was analyzed by flow cytometry using anti–HLA-A2 MAb (A2.28) and anti-HLA class I antibody (W6/32).

†Values represent the percentage of cells reactive with the antibodies listed. Numbers in parentheses are the mean fluorescence intensity as determined in relative log units.

‡Results are expressed in percent specific lysis in a 12-hour ^{111}In release assay at effector-to-target ratio of 50:1.

§Statistically significant lysis ($P<.01$, paired t test).

The finding reported here that the administration of rV-CEA can elicit a T-cell response to a defined 9-mer epitope on CEA leads to potential second-generation protocols. One of the limitations on the use of a vaccinia recombinant vaccine is that it elicits a strong antibody response to the vaccinia virus (*13*). This in turn, prohibits numerous boosts with the recombinant vaccinia, due to the anti-vaccinia antibody inhibition of local virus spread. Indeed, this boost in anti-vaccinia antibody, seen in the phase I trial with rV-CEA, may be a limitation in the sole use of rV-CEA as immunogen.

Now that a specific epitope on the CEA molecule has been identified, subsequent studies in HLA-A2 patients can be designed in which one or two rV-CEA administrations are followed by several administrations of the CAP-1 peptide (amino acid 571-579) in adjuvant or liposomes as boosts. Moreover, protocols involving the use of rV-CEA and recombinant CEA protein, anti-idiotype MAb (as surrogate antigen for CEA), or other viral vectors may be considered. It should be pointed out that the T cells recognizing the CAP-1 peptide do not recognize the corresponding sequence on NCA. In addition, the specificity of V24T was demonstrated by the induction of TNF-α secretion by incubation of V24T with autologous B cells pulsed with CAP-1 but not with CAP-9 or CAP-10 peptides.

An alternative approach would be to expand in vitro the CTL lines derived from PBLs of rV-CEA–immunized HLA-A2 patients by pulsing with the CAP-1 peptide plus IL-2. These antigen-specific CTLs could then be adoptively transferred to the autologous patient. The adoptive transfer of antigen-specific CTL could conceivably be followed by administration of the CAP-1 peptide in adjuvant or a liposomal formulation to further expand the CEA-specific CTL population in vivo. Perhaps fewer cells of this type need to be adoptively transferred than in tumor-infiltrating lymphocyte protocols (*45*), since these antigen specific T cells are expanded and activated by a known 9-mer epitope.

The broad use of peptide CAP-1 as an immunogen is an intriguing issue. We saw evidence of T-cell growth and the establishment of CTL lines from post-immunization PBLs from three of three HLA-A2 individuals. Unexpectedly, we also saw indications of cell growth in post-immunization PBLs from two individuals who were negative for the A2 allele. In both of these cases, however, T-cell growth was not seen until after three cycles of stimulation with peptide and IL-2. If in fact these are MHC-restricted T-cell responses, one must postulate that the peptide designed to bind to HLA-A2 molecules can also bind to other HLA class I or II molecules. In principle, this phenomenon could arise from the following scenarios: (*a*) alternate class I or II molecules share consensus motifs with HLA-A2 or (*b*) the CAP-1 peptide fortuitously contains sequences capable of binding more than one HLA class I or II antigen, i.e., contains multiple consensus motifs. The current literature on MHC restriction offers examples of both scenarios. For example, HLA-

A3 and HLA-A11 molecules are both known to prefer 9-mer peptides with valine at position 2 and lysine at the C terminal (*46*). An example of the second situation is the specific immunity generated in mice of different genetic backgrounds to a defined viral epitope of lymphocytic choriomeningitis virus (*47*).

Less controversial, but equally intriguing, is the suggestion that T-cell growth was observed in vitro in HLA-A3 PBL (patient Vac32) by stimulation with a peptide that also fits the A3 consensus motif. If this HLA-A3 stimulation turns out to be the case, evidence is provided that rV-CEA can induce MHC-restricted CTL responses in individuals with various haplotypes.

The area of T-cell immunotherapy that is now emerging involves at least two vital parts. The first is the identification of the target antigen/epitope in the context of known MHC molecules in terms of recognition by T cells. The second vital step involves T-cell activation and clonal expansion. Thus, studies are now under way to better activate T cells recognizing specific human epitopes. These studies will involve the use of cytokines and T-cell costimulatory molecules such as B7.1. In a recent study (*48*), we have demonstrated that the murine B7.1 and B7.2 molecules can each be placed into a vaccinia virus vector to enhance the immunogenicity of murine carcinoma cells in an experimental model. Studies are currently under way in an experimental model to coadminister rV-CEA with rV-B7 recombinants to enhance the immunogenicity of CEA; preliminary results appear to be promising.

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Notes

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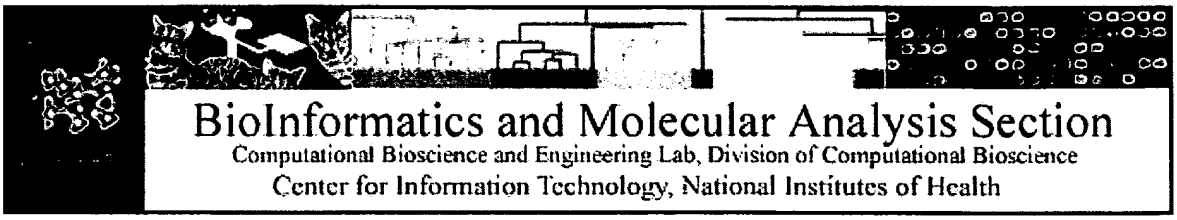

HLA Peptide Binding Predictions

Function: Rank potential 8-mer, 9-mer, or 10-mer peptides based on a predicted half-time of dissociation to HLA class I molecules. The analysis is based on coefficient tables deduced from the published literature by Dr. Kenneth Parker, Children's Hospital Boston (email: kenneth.parker@childrens.harvard.edu).
Another web site for predicting which peptides bind to MHC molecules is SYFPEITHI, developed by Hans-Georg Rammensee's lab.

Analysis Options: **HLA molecule** **n-mers**

A1
A_0201
A_0205
A24
A3

9

Results Limited by: ◉ **Explicit Number** ○ **Predicted $T_{(½)}$ >=**

20 100

Please enter or paste an AA sequence to analyze (most formats accepted):

☑ Echo input sequence (generally recommended)

submit reset

Credits: WWW implementation by Ronald Taylor of BIMAS / CBEL / CIT / NIH

If you use results from this analysis in published research, please cite:

Parker, K. C., M. A. Bednarek, and J. E. Coligan. 1994. Scheme for ranking potential HLA-A2 binding peptides based on independent binding of individual peptide side-chains. J. Immunol. 152:163.

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